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Examining the Relationship between Urinary Pathogens, Antibiotic Exposure and Urolithiasis

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Abstract

Urinary stone disease is a highly prevalent urological condition; however, the pathophysiology remains poorly understood. Growing evidence suggests a relationship between urinary tract infections, antibiotic exposure and the development of urolithiasis. In this project, we utilized a dietary *Drosophila melanogaster* model and a calcium oxalate crystal adhesion assay to further investigate the impact of a urinary pathogen and antibiotics on calcium stone formation. We demonstrated that both a non-urease producing strain of *Escherichia coli* and the antibiotics, ciprofloxacin and trimethoprim-sulfamethoxazole, increased calcium stone formation. In addition, our preliminary work suggests that biomineralization agents such as osteopontin and zinc appear to be implicated in this process. Further research is required to better delineate the mechanisms involved and to translate these findings into potential therapeutic and prevention strategies for human stone disease including antibiotic stewardship, anti-oxidants, probiotics, and microbiome modification.

Keywords

antibiotics, calcium oxalate, ciprofloxacin, crystal adherence, *Drosophila melanogaster*, *Escherichia coli*, inflammation, microbiota, osteopontin, renal epithelial cells, trimethoprim-sulfamethoxazole, urinary pathogens, urinary tract infection, urolithiasis, zinc

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Chapter 1

1 Introduction

Urinary stone disease is a highly prevalent condition which affects approximately 10% of the adult population, and has been shown to have increased in incidence significantly over the past twenty years[1]. While mortality resulting from urinary stone disease is rare with modern medical treatment, the condition still results in significant morbidity including acute renal colic, chronic kidney disease and urosepsis. In addition, urolithiasis has a high recurrence rate of approximately 50% at ten years without preventative treatment[2]. As a result, urinary stone disease places a tremendous burden on the health care system with an estimated 185,000 hospitalizations, 2 million outpatient visits, and 2.1 billion dollars expended in the United States annually[3,4]. Current dietary and pharmacological treatment strategies have led to only a modest improvement in stone recurrence rates. Consequently, an improved understanding of the pathophysiology of stone development is paramount to developing more efficacious prevention strategies[5,6,7].

1.1 Epidemiology of Urolithiasis

Urinary stone disease is a common condition worldwide, with incidence rates ranging from 7-13% in North America, 5-9% in Europe, and 1-5% in Asia[1]. In addition, multiple reports have demonstrated an increasing prevalence of stone disease across the globe over the past several decades. The prevalence rate was shown to have risen a dramatic 37% in American adults from 1976 to 1994[8]. Comparable increases have also been observed in Japan and Iceland, where the annual incidence has been observed to triple. In Japan, the annual incidence of first-episode urinary stone disease rose from 54.2 to 134.0 per 100,000 from 1965 to 2005[9]. Similarly, the incidence of stone disease in Iceland increased from 7 to 24 per 100,000 over a twenty-four-year period[10]. Multiple theories have been proposed to explain this trend including the increased use of abdominal imaging leading to higher rates of diagnosis for asymptomatic stones, and global warming resulting in expansion of areas at high risk for dehydration and subsequent stone formation[1]. In addition, the dramatic rise of conditions associated with stone formation including obesity, diabetes mellitus, and metabolic syndrome are likely also contributing to the rising incidence of urolithiasis[1]. Along with this, changing dietary patterns which include decreased water and calcium consumption, and increased intake of dietary sodium, animal protein and sweetened soft drinks has also been linked with stone disease[1].

However, the rising incidence of stone disease has been most pronounced in populations which have typically been thought to be at lower risk for urolithiasis, such as women and children. In the latter, the incidence of stone disease has increased a startling 4%

annually over the past twenty-five years[11]. Similarly, the incidence of stone disease has been observed to rise an average of 1.9% per year from 1970 to 2000 in American women[12]. In addition, multiple reports have demonstrated a narrowing of the gender gap in the prevalence of stone disease between males and females[12,13,14]. A recent report examining data from the National Health and Nutrition Examination Survey (NHANES) from 2007 to 2012 failed to show any differences in stone prevalence between males and females; suggesting that the long held gender gap in stone disease may be closing[15]. While the substantial increase in urolithiasis has been partially attributed to the significant rise of obesity and diabetes, the potential causes behind the changing demographics of stone disease has yet to be fully elucidated[1].

1.2 Clinical Presentation of Urolithiasis

Pain is the most common symptom associated with urolithiasis and results from the obstruction of urine by the stone. The pain is typically colicky in nature and may be accompanied by nausea and vomiting. Distention of the renal capsule from hydronephrosis results in nausea and vomiting due to the shared splanchnic innervation of the gastrointestinal tract and the renal capsule[16]. Patients will typically experience unilateral flank pain which radiates to the ipsilateral groin with calculi within the upper urinary tract. As the stone migrates more distally into the lower ureter, the pain may radiate to the testicle or labium and be associated with irritative voiding symptoms such as urinary urgency and frequency[16].

Pain is frequently accompanied with hematuria and up to 85% of patients will experience either gross or microscopic hematuria during their acute stone episode[17]. In addition, a number of patients with stone disease may also present with painless hematuria resulting in diagnosis.

Patients may occasionally present with renal dysfunction secondary to urinary obstruction in the setting of a solitary kidney, bilateral obstructing calculi, pre-existing renal insufficiency, or prolonged obstruction. Furthermore, patients can also present with fever or urosepsis resulting from a urinary infection combined with urinary obstruction. Finally, an increasing number of patients are now being diagnosed with asymptomatic urolithiasis detected incidentally on abdominal imaging for unrelated indications given the widespread availability of multiple imaging modalities[18].

1.3 Pathogenesis of Urolithiasis

Despite years of extensive investigation, the pathogenesis and physiochemistry mechanisms of stone formation remain incompletely understood[19]. The formation of a urinary calculi is a complex cascade of events that begins with supersaturation of the urine with stone-forming solutes; following this, crystals become retained within the kidney at anchoring sites allowing for further crystal growth and aggregation[20]. Supersaturation of the urine with lithogenic solutes is influenced by several etiological factors including the increased production or excretion of the specific solute and urinary pH which modifies the solubility of the solute within the urine[21]. Once there is

supersaturation of a solute, homogenous nucleation occurs forming a crystal nuclei[21]. The crystal nuclei are small and can often be destabilized by stone inhibitors in the urine such as citrate, magnesium, and nephrocalcin; which result in the dissolution of the nuclei[22]. However, stone promoters within the urine can act to stabilize the nuclei allowing for further crystal growth and aggregation[22].

Fixed particle growth theory postulates that stone crystals bind to an anchoring site within the kidney resulting in prolonged exposure of the crystal to supersaturated urine thereby facilitating further crystal growth and aggregation[23]. While the mechanism behind crystal fixation is not fully understood, it has been proposed that high levels of urinary oxalate lead to renal tubular epithelial cell damage and result in increased adherence of calcium oxalate (CaOx) crystals[23]. It is thought that oxalate-induced cell injury is mediated through oxidative stress and reactive oxygen species (ROS). In addition, oxidative stress has been suggested as a potential mechanism to explain the association between stone disease and other conditions associated with high levels of oxidative stress such as diabetes and metabolic syndrome[24].

1.4 Classification of Urolithiasis

Urolithiasis is most commonly classified according to stone composition and includes calcium-based, uric acid, struvite, and cystine stones (table 1)[25]. The majority of calculi are heterogeneous in composition reflecting the complex physiochemistry involved in stone formation. Calcium-based stones are the most prevalent stone type (80%) and are typically comprised of CaOx (70%) in either the monohydrate (COM) or dihydrate

(COD) form[25]. Calcium phosphate encompasses a much smaller percentage of calcium-based stones (10%). In addition, calcium stones may also contain small amounts of hydroxyapatite and uric acid. Uric acid stones are the second most common stone composition (7%) and may contain small amounts of hydroxyapatite and struvite. Struvite calculi (7%), which are magnesium ammonium phosphate stones, are typically associated with urinary infection with urease-splitting organisms. Cystine stones, which are a rare stone type (3%), are formed as a result of the autosomal recessive genetic disorder cystinuria. Finally, there are a number of other rare stone types such as xanthine and multiple drug related stones (triamterene, indinavir) which can occur[25].

Table 1: Classification of urolithiasis by stone composition and occurrence[25].

Stone Composition	**Occurrence (%)[25]**	**Stone Constituents**
Calcium oxalate	60	Monohydrate (Whewellite) Dihydrate (Weddellite)
Calcium phosphate	20	Brushite Hydroxyapatite
Uric acid	7	Uric acid Monosodium urate
Struvite	7	Ammonium-magnesium phosphate
Cystine	3	Cystine
Miscellaneous	<1	Xanthine Triamterene Indinavir

1.4.1 Calcium Oxalate

Despite being the most commonly encountered stone type, the etiology of CaOx urolithiasis is poorly understood and likely results from the complex interplay of many genetic and environmental factors. Many conditions and metabolic abnormalities have been identified which predispose to CaOx stone formation including hypercalciuria, hyperoxaluria, hyperuricosuria, and hypocitraturia. However, a significant proportion of CaOx stone formers are idiopathic highlighting our incomplete understanding of the pathophysiology of calcium stone disease.

1.4.1.1 Hypercalciuria

Hypercalciuria is a common metabolic disturbance in calcium stone formers and is identified in 35-65% of patients[26]. Hypercalciuria is defined as excretion of greater than 4 mg/kg/day of calcium within the urine and results in an increased risk of stone formation through increased supersaturation of the urine and reduced activity of urinary stone inhibitors[27]. The causes of hypercalciuria can be broadly categorized as absorptive, resorptive, renal leak and idiopathic; however, hypercalciuria likely results from multiple interrelated physiological disturbances. Absorptive hypercalciuria occurs when there is increased intestinal absorption of calcium which is matched by a proportional increase in renal calcium excretion by the kidney. The mechanism behind absorptive hypercalciuria has not been fully elucidated; however, it has been linked to a number of genetic

abnormalities which alter vitamin D activity such as mutations in the CYP24A1 or vitamin D receptor (VDR) gene[28,29].

Renal leak hypercalciuria occurs when the renal tubule fails to absorb an adequate amount of calcium resulting in elevated urinary calcium levels and secondary hyperparathyroidism[30]. Similarly, the precise cause of renal leak hypercalciuria remains unknown. It has been associated with several genetic mutations which affect a number of transport channels within the tubule[31,32].

Resorptive hypercalciuria is an infrequently encountered cause of hypercalciuria which most commonly results from primary hyperparathyroidism. Only approximately 5% of cases of nephrolithiasis are associated with primary hyperparathyroidism[33]. In primary hyperparathyroidism excessive parathyroid hormone (PTH) secretion from a parathyroid adenoma results in elevated serum calcium and thus increased urinary excretion of calcium. The elevated serum calcium results from excessive bone resorption and increased renal synthesis of $1,25(OH)_2D_3$ which in turn increases intestinal absorption of calcium. Interestingly, only 25-55% of patients with primary hyperparathyroidism will be affected with urolithiasis, suggesting that the etiology of stones requires more than isolated hypercalciuria and remains incompletely understood[34]. There are additional rarer causes of resorptive hypercalciuria including malignancy, granulomatous diseases, thyrotoxicosis and vitamin D toxicity[35].

1.4.1.2 Hyperoxaluria

In addition to hypercalciuria, a proportion of CaOx stone formers (2-15%) will have hyperoxaluria, which is defined as a urinary oxalate excretion of greater than 40mg/day[26]. Elevated urinary oxalate levels contribute to the supersaturation of CaOx and reduced activity of stone inhibitors; however, oxalate may further propagate crystal adherence and growth by causing renal tubular damage through oxidative stress and ROS[36]. Antioxidant therapy has been demonstrated to prevent CaOx crystal deposition in renal urothelium both *in vitro* and in rat kidneys[36,37]. The causes of hyperoxaluria can be categorized as either primary, enteric, or dietary forms.

Primary hyperoxaluria is a rare autosomal recessive genetic condition in glyoxylate metabolism which results in the excessive production and subsequent urinary excretion of oxalate. In this condition, the normal conversion of glyoxylate to glycine is prevented and thus glyoxylate is preferentially converted to oxalate. The markedly elevated levels of urinary oxalate result in the supersaturation of CaOx within the urine and subsequent stone formation. In addition, CaOx crystals are internalized into tubular cells and then extruded into the renal interstitium resulting in nephrocalcinosis[38].

Dietary hyperoxaluria occurs from the over indulgence of foods rich in oxalate such as spinach, rhubarb, nuts, chocolate and tea. Exogenous oxalate contributes to 24 to 42% of excreted urinary oxalate[39]. In addition, diets low in calcium intake can result in increased intestinal oxalate absorption due to the reduced intestinal binding of oxalate. Finally,

ascorbic acid is converted to oxalate and can also increase urinary oxalate levels, and has been associated with increased stone formation[40,41].

The most common cause of acquired hyperoxaluria is the enteric form. This occurs when malabsorptive states result in fatty acid saponification with cations such as calcium and magnesium, thereby reducing the intestinal binding of calcium and oxalate and increasing intestinal oxalate absorption[42]. In addition, the poorly absorbed fatty acids have been shown to increase colonic permeability to oxalate resulting in a further increase in oxalate absorption[43,44]. Conditions associated with enteric hyperoxaluria include chronic diarrheal states, prior small bowel resection, intrinsic disease, and jejunoileal bypass[45]. The prevalence of enteric hyperoxaluria is especially important in patients undergoing malabsorptive bariatric surgery as these patients commonly have a number of other baseline metabolic derangements that put them at higher risk for stone formation. Unfortunately, this is becoming an increasingly encountered clinical scenario with the rise of the obesity epidemic.

The intestinal absorption of oxalate has also been associated with the bacterium *Oxalobacter formigenes* which is a gram-negative, obligatory anaerobic bacterium which colonizes the intestinal tract of humans[46]. *Oxalobacter formigenes* utilizes oxalate as its primary carbon source through the oxalyl-CoA decarboxylase enzyme, and as a result it has been theorized that colonization with *O. formigenes* can impact the intestinal absorption of oxalate and thus urinary oxalate excretion[47]. Previous studies have demonstrated colonization with *O. formigenes* resulted in reduced urinary oxalate excretion, reduced plasma oxalate, and a lower risk of developing CaOx stone disease[48-51]. Prolonged antibiotic use appears to negatively impact *O. formigenes* colonization and

may represent one of the mechanisms by which antibiotics affect stone formation. Patients with cystic fibrosis and *Helicobacter pylori* infection who are often treated with prolonged courses of antibiotics have been demonstrated to have an absence of *O. formigenes* colonization and as a result elevated urinary oxalate levels[52,53].

1.4.1.3 Hyperuricosuria

Hyperuricosuria defined as a urinary uric acid level exceeding 600mg per day has also been associated with an increased risk of CaOx stone formation and is found in up to 10% of calcium stone formers[54]. It is thought that elevated levels of urinary uric acid increase CaOx stone formation through the process of heterogeneous nucleation by providing a nidus for further crystal aggregation and growth[55]. Uric acid has also been shown to reduce the effectiveness of multiple urinary stone inhibitors[56]. The most common cause of hyperuricosuria is elevated dietary purine intake. A number of other conditions associated with altered purine metabolism such as Lesch-Nyhan Syndrome and gout; as well as conditions associated with rapid tissue turnover including myeloproliferative disorders, and multiple myeloma can also result in hyperuricosuria and uric acid stone formation[57].

1.4.1.4 Hypocitraturia

Hypocitraturia occurs in approximately 10% of calcium stone formers and is defined as a urinary citrate level less than 320 mg/day[58]. Citrate acts as an important inhibitor of stone formation through several different mechanisms. It has been shown to reduce urinary saturation of calcium, prevent spontaneous nucleation of CaOx, inhibit aggregation and growth of CaOx crystals, and enhance the effect of other stone inhibitors[59-63]. Urinary citrate excretion is primarily determined by acid-base balance and metabolic acidosis will reduce urinary citrate levels through increased tubular reabsorption and decreased citrate synthesis in the peritubular cells[64]. Hypocitraturia can result from a number of disease states including chronic diarrheal states, excessive animal protein intake, medications such as thiazide diuretics and angiotensin-converting enzymes, and distal renal tubular acidosis (RTA).

In addition, there are a number of other abnormalities which have been associated with an increased risk of CaOx stone disease such as low urine pH, hypomagnesuria and low urine volume. A significant proportion of CaOx stone formers will have multiple different metabolic abnormalities detected, and approximately 3% of patients will demonstrate no abnormality on their metabolic evaluation[65]. The overall pathogenesis of CaOx stone formation is not completely understood and is likely a complex process involving the interplay of multiple different mechanisms.

1.4.2 Uric Acid

Uric acid is formed as a byproduct of purine metabolism and can accumulate significantly within human blood and urine resulting in uric acid stones[66]. The formation of uric acid stones is determined by urinary pH, hyperuricosuria, and low urine volumes. Uric acid can be present as either free uric acid or urate salt, depending on the pH. Urate salt is approximately twenty times more soluble than free uric acid and consequently, the risk of uric acid stone formation is highly dependent on urine pH[67]. For instance, at a urinary pH of 5 the supersaturation of uric acid is easily reached with the excretion of normal amounts of uric acid; however, increasing the urinary pH to 6.5 allows for significantly higher concentrations of uric acid to remain soluble within the urine[67]. Conditions associated with low urine pH and low urine volumes such as intestinal malabsorption and chronic diarrheal states are at increased risk for uric acid urolithiasis due to a loss of bicarbonate and concentrated acidic urine[68]. In addition, type two diabetes and metabolic syndrome has also been demonstrated to be a risk factor for uric acid stone formation by reducing ammoniagenesis and lowering urinary pH[69]. Hyperuricosuria can also result in the formation of uric acid urolithiasis through extremely elevated levels of urinary uric acid which crystallize regardless of urinary pH. This can be found in patients with gout, myeloproliferative disorders, and tumor lysis syndrome.

1.4.3 Struvite

Struvite stones which are classically referred to as infection stones are composed primarily of magnesium ammonium phosphate and occur following urinary infection with a urease splitting organism. The process of urea-lysis provides an alkaline environment as well as sufficient concentrations of carbonate and ammonia which allow the production of struvite stones[70]. Urea, which is a normal constituent of urine, is hydrolyzed to ammonia by bacterial urease[70]. This results in alkaline urine which favors the formation of ammonium and further increases the urinary pH[70]. This promotes the formation of carbonate and phosphate which along with physiologic concentrations of magnesium provide the constituents for struvite formation (figure 1)[70].

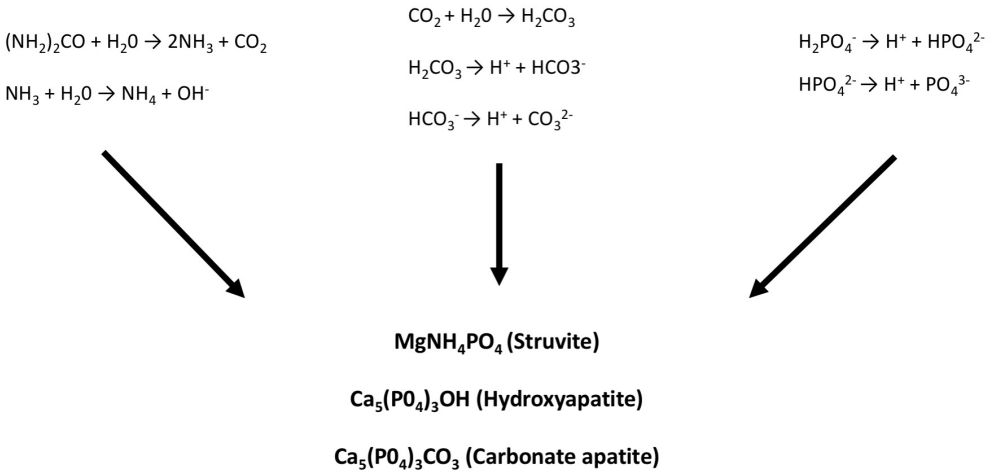

Figure 1: Chemical reactions leading to the formation of infection based urinary calculi.

The most commonly encountered urease-splitting pathogens include *Proteus, Klebsiella, Pseudomonas* and *Staphylococcus* species; however, a number of gram-positive and gram-negative bacteria as well as yeast species have demonstrated the ability to synthesize urease (table 2)[71]. *Escherichia coli* which is a common organism in urinary tract infections (UTIs) only rarely produces urease[72]. In addition to the ability to synthesize urease, bacteria may further propagate stone formation by causing urothelial damage allowing for increased bacterial colonization and crystal adherence[73,74]. Furthermore, certain bacteria have also been observed to reduce the activity of certain urinary stone inhibitors such as urokinase[75].

Table 2: Urease producing organisms.

Gram-negative	*Proteus rettgeri*
	Proteus vulgaris
	Proteus mirabilis
	Proteus morganii
	Providencia stuartii
	Haemophilus influenzae
	Bordetella pertussis
	Bacteroides corrodens
	Yersinia enterocolitica
	Brucella species
	Klebsiella pneumoniae
	Klebsiella oxytoca
	Serretia marcescens
	Haemophilus parainfluenzae
	Bordetella bronchiseptica
	Aeromonas hydrophila
	Pseudomonas aeruginosa
	Pasteurella species
Gram-positive	*Flavobacterium* species
	Staphylococcus aureus
	Micrococcus species
	Corynebacterium ulcerans
	Corynebacterium renale
	Corynebacterium ovis
	Corynebacterium hofmannii
	Staphylococcus epidermidis
	Bacillus species
	Corynebacterium murium
	Corynebacterium equi
	Peptostreptococcus asaccharolyticus
	Clostridium tetani
	Mycobacterium rhodochrous group
Mycoplasma	T-strain *mycoplasma*
	Ureaplasma urealyticum
Yeast	*Cryptococcus*
	Rhodotorula
	Sporoblomyces
	Candida humicola
	Trichosporon cutaneum

Struvite stones are most commonly observed in patients at higher risk for urinary infections including females, the elderly, premature infants, and patients with urinary stasis from urinary tract anomalies, urinary diversions, or neurogenic urinary tract dysfunction.

1.4.4 Cystine

Cystinuria is an inherited autosomal recessive disorder which results in the excessive urinary excretion of dibasic amino acids including cystine, lysine, ornithine, and arginine[76]. Mutations in the dibasic amino acid transporters SLC3A1 and LSC7A9 result in impaired proximal tubular reabsorption of cystine, and the poor solubility of cystine within the urine leads to the formation of cystine stones[77,78]. The solubility of cystine and thus the formation of cystine stones depend on multiple factors including cystine concentration, urine pH, ionic strength, and the presence of macromolecules. However, because there is no specific inhibitor of cystine crystallization within the urine supersaturation of cystine and subsequent cystine stone formation occur readily in physiologic conditions[79]. Patients with cystinuria typically present early in life and have multiple episodes of recurrent stone formation.

1.5 Treatment of Urolithiasis

There exist multiple potential treatment options for stone disease and often the optimal treatment decision is complex and based on a multitude of patient and stone factors. Potential treatment strategies can be broadly divided into surgical and non-surgical options. Non-surgical strategies include active surveillance and expectant management with or without medical expulsive therapy (MET). Small, non-obstructive, asymptomatic calyceal stones can be managed conservatively with active surveillance[80]. In general, the first line treatment for small ureteric stones is a trial of expectant management as the majority of these stones will pass spontaneously[80]. MET with α-blockers or calcium channel blockers has been shown to increase the likelihood of spontaneous ureteric stone passage[81]. Indications for surgical intervention include the presence of infection, an obstructed solitary kidney or bilateral obstruction, intractable pain or nausea and vomiting, and failed expectant management.

Surgical treatment options include urinary drainage with a stent or nephrostomy tube, shockwave lithotripsy (SWL), ureteroscopy, or percutaneous nephrolithotomy (PCNL). SWL is the procedure with the least morbidity and lowest complication rate; however, ureteroscopy is associated with a higher stone free rate following a single procedure[80]. The efficacy of SWL is dependent on a number of patient and stone factors and has been demonstrated to be less effective for mid or distal ureteric calculi, larger stones within the lower pole of the kidney (greater than 1cm), patient obesity, and hard stone compositions (COM, calcium phosphate, and cystine)[80]. PCNL is typically reserved for large renal

stone burdens (greater than 2cm), large lower pole stones with unfavourable anatomy, or anatomical abnormalities that preclude other treatment options[80].

Urolithiasis is a highly recurrent disease and approximately 50% of patients will form a recurrent stone within ten years of their initial stone episode[2]. Prevention strategies include both general dietary and lifestyle recommendations as well as specific medical prophylaxis based on stone composition and abnormalities identified on in-depth metabolic evaluations. General lifestyle and dietary measures include increasing fluid intake to achieve a daily urine output of 2.5L, dietary calcium intake of 1000-1200 mg/day ideally with meals, treatment of documented vitamin D deficiency, moderation of animal protein intake, avoidance of purine rich foods, limiting daily sodium intake to 1500 mg/day, having a diet rich in fiber, fruits and vegetables, and maintaining a healthy body weight[82]. In addition, there are a number of medical therapies which are utilized to correct specific abnormalities which are identified on a metabolic evaluation. These medications include thiazide diuretics for the treatment of hypercalciuria, urinary alkalinization with alkali citrates, xanthine oxidase inhibitors for the treatment of hyperuricosuria and thiol binding agents for cystine stone formers[82]. Unfortunately, current preventative treatment strategies have only resulted in a modest reduction of stone recurrence rates. In addition, no new therapies for stone prevention have been developed since the 1980's when potassium citrate was introduced[83]. Consequently, an improved understanding of the pathophysiology behind stone formation is critical in order to allow for the development of new treatment and prevention strategies.

1.6 Models of Stone Disease

Despite urinary stone disease being a common condition, which results in significant patient morbidity and associated health care costs, our understanding of the pathophysiological mechanisms underlying stone formation remain limited. An important limitation of stone disease research is the lack of a suitable pre-clinical research model. The ideal model for investigating urinary stone disease should result in consistent stone formation and recapitulate the physiology of the human nephron. In addition, the model should allow for the manipulation of stone formation in order to investigate potential therapeutic agents. Finally, the model should be simple, practical, inexpensive, and high-throughput.

Previous animal models of stone disease include murine, canine and porcine models; however, these all carry significant limitations[84-87]. The rat model has historically been the most commonly utilized for studying calcium stone disease. Spontaneous stone formation does not occur within the rat and must be induced through the administration of lithogenic agents which produce hyperoxaluria such as sodium oxalate, ammonium oxalate, hydroxy-L-proline, ethylene glycol and glycolic acid[86,88-92]. This does not accurately recapitulate the metabolic milieu present in human urolithiasis where other metabolic derangements other than hyperoxaluria are present[88]. In addition, stone formation in this model occurs in an unpredictable fashion with only a minority of the experimental animals actually forming stones following a prolonged timeline of up to

four weeks[91,93-96]. Many of these lithogenic agents are often nephrotoxic and lead to renal failure and death of the experimental model[97]. The majority of the formed calcium oxalate crystals are non-adherent to the renal epithelium and are washed out in the urine; this fails to accurately represent stone formation in humans as crystal adherence to the renal epithelium is a critical initial event and makes measurement of stone deposition difficult[98]. The rat model does not allow for live *in vivo* imaging to visualize stone burden and the only method to accurately measure stone burden is to sacrifice the animal. This results in an unnecessary loss of animals, increased costs, and limits the ability to investigate potential therapeutic targets.

Given the significant costs and resources required for the murine model of stone disease, larger scale studies are not feasible. Consequently, the majority of previous studies include small sample sizes which limits the validity of the results. The current available animal models for urolithiasis have significant limitations which has prompted the development of newer novel models for stone disease.

1.6.1 *Drosophila melanogaster* Stone Model

Drosophila melanogaster (DM) has been widely utilized as a versatile model for numerous human conditions including diabetes and renal disease[99,100]. The *Drosophila* genome is fully sequenced and has been demonstrated to be highly conserved with the human genome with over 70% of DM genes having human homologs[101]. Extensive prior research involving the DM genome has led to the development of exhaustive genome

databases and microarray expression data[102,103]. In addition, the DM genome is easily modifiable using well-established genetic tools making it an invaluable research model[104].

The DM renal system is composed of two components, the nephrocytes and the Malpighian tubules and functions as a rudimentary human kidney. The nephrocytes are the functional equivalent of human podocytes within the glomerulus and function to filter the insect hemolymph[105]. The DM have four Malpighian tubules, which are divided into anterior and posterior segments, and join into a common ureter before draining into the gut at the junction between the mid and hindgut[106]. The Malpighian tubules serve as the functional equivalent of the human convoluted tubule have been well characterized in regard to solute transport and excretion mechanisms[106]. A diagrammatic comparison of the human and DM kidney is illustrated in figure 2.

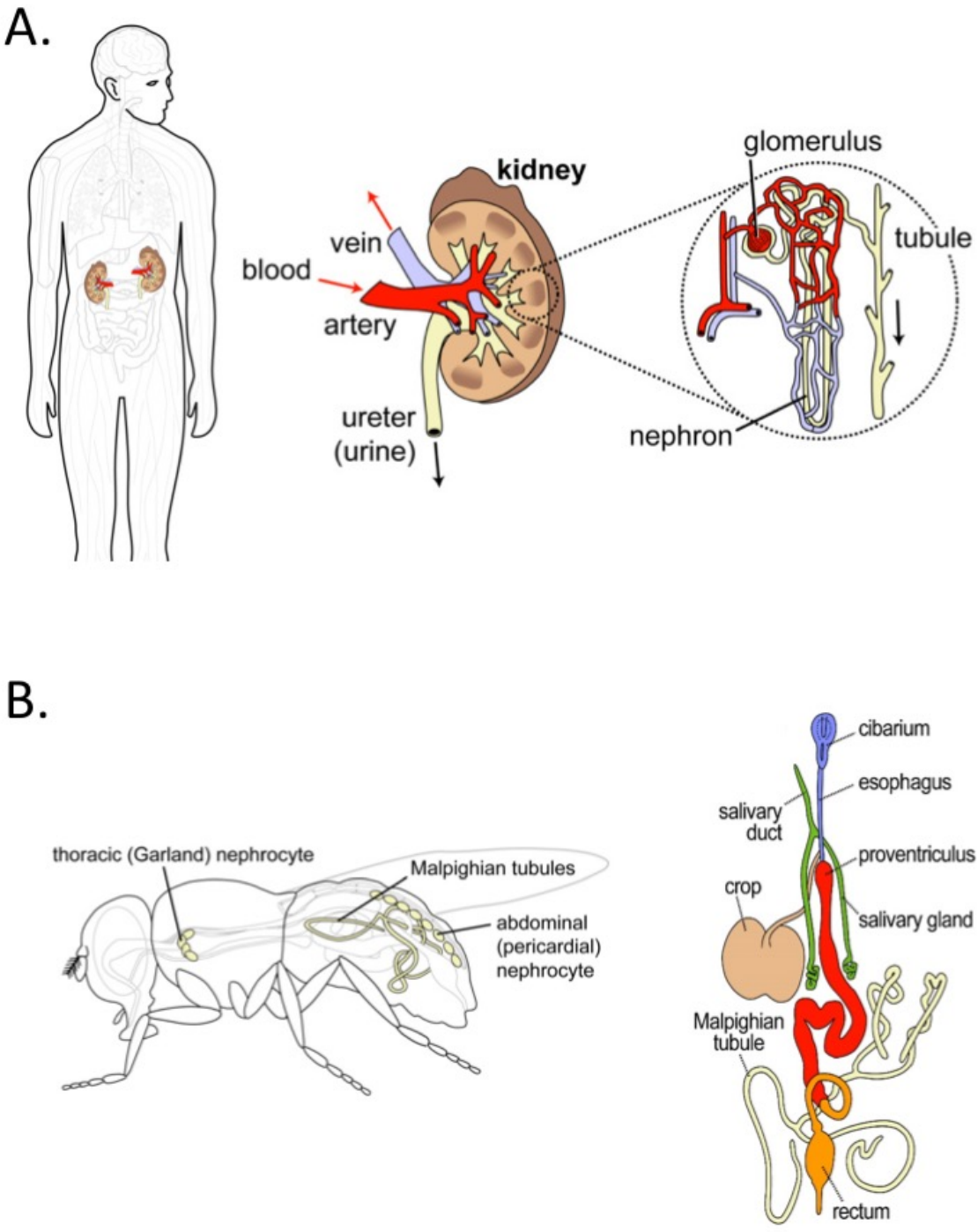

Figure 2: Schematic representation of human and *Drosophila melanogaster* urinary excretion systems[107].

1.6.2 Crystal Adhesion Assay

The formation of calcium oxalate stones in humans represents a complex and multi-factorial metabolic process of which the complete pathophysiology remains unknown. While animal models are valuable for investigating the overall process of stone formation and potential therapeutic agents, *in vitro* models can allow for more specific examination of the mechanisms involved in stone formation.

Examination of CaOx crystallization, adherence, aggregation, and growth with *in vitro* assays have been well utilized to delineate the mechanisms involved in stone formation[108-112]. Much of this research has formed our current understanding of the pathophysiological mechanisms involved in urinary stone disease. While there are inherent limitations to *in vitro* models; they can provide a practical, inexpensive and informative method to investigate mechanistic pathways and examine the interplay between different factors.

The adherence of CaOx crystals to renal epithelial cells is a critical initial step in the formation of urinary calculi which allows for further aggregation and growth of the crystals[113]. Without this step the crystals would pass through the urinary system without difficulty[113]. *In vitro* assays examining the adherence of CaOx crystals to renal epithelial cells have previously been developed and utilized to examine stone formation and the effect of potential stone inhibitors[108-113]. This assay serves as a useful platform to not only understand the mechanisms involved but also to identify potential therapeutic targets

in a high throughput fashion, which can then be further examined with *in vivo* and human models.

1.7 Objectives and Aims

Objective 1: Determine whether CaOx stone formation in a *Drosophila melanogaster* model is affected by a non-urease producing urinary pathogenic bacterial isolate and/or by the addition of different antibiotic classes associated with increased human urolithiasis occurrence.

Epidemiological studies have demonstrated a correlation between both urinary pathogens and antibiotic exposure on the development of urolithiasis. In order to further examine potential causation, we investigated the effect of a non-urease producing strain of *E. coli* and both ciprofloxacin and trimethoprim-sulfamethoxazole (TMP-SMX) on the development of CaOx stones in a *Drosophila* model.

Objective 2: Determine whether CaOx crystal adherence is affected by a non-urease producing urinary pathogenic bacterial isolate and/or by the addition of different antibiotic classes associated with increased human urolithiasis occurrence.

In order to further explore potential mechanisms by which both urinary pathogens and antibiotics may affect stone formation, we will determine the effect of *E. coli* and both ciprofloxacin and TMP-SMX on the adherence of CaOx crystals to renal epithelial cells.

Objective 3: Determine the effect of osteopontin (OPN) on CaOx crystal adherence to renal epithelial cells.

Osteopontin, a known potent inhibitor of CaOx urolithiasis, has now also been shown to play an important role in gastrointestinal inflammation and have an inhibitory effect on endodontic bacteria. Given this, we aimed to further study this urolithiasis-preventing protein in the context of its influence with the newly described presence of bacteria in the kidney[8, 9]. We will determine the effect of *E. coli* and OPN on the adherence of CaOx crystals to renal epithelial cells.

Objective 4: Determine the effect of zinc (Zn) chloride on CaOx crystal adherence to renal epithelial cells.

Given that Zn is known to play a vital role in bacterial pathogenesis and immune modulation and has also been implicated as an early nidus for the mineralization process of urinary stones, we would like to further explore its role in the relationship between urinary bacteria and urolithiasis[10, 11, 12]. The effect of Zn and *E. coli* on the adherence of CaOx crystals to renal epithelial cells will be determined.

Our hypothesis is that urinary bacteria and the antibiotics, ciprofloxacin and trimethoprim-sulfa methazole, potentiate CaOx stone formation and crystal adhesion by through the regulation of OPN and Zn transport mechanisms.

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Chapter 2

2 Effect of Antibiotics and a *Escherichia coli* Urinary Infection Isolate on Calcium Urolithiasis

Urinary stone disease is a highly prevalent condition with a dramatically rising incidence; however, the increasing incidence of stone disease has been most pronounced in populations typically at lower risk for urolithiasis such as women and children. The cause behind the changing demographics of stone disease has yet to be completely understood. To further elucidate this, the potential effect of a non-urease producing bacteria and treatment with antibiotics on the formation of CaOx stones was investigated in well-established stone models. These included the DM fly model, and an assay of CaOx crystal adherence to renal epithelial cells[1,2].

2.1 Introduction

2.1.1 Urinary Bacteria and Urolithiasis

The casual association between urease-splitting uropathogens and struvite stone formation is well established. There is increasing evidence that suggests a relationship between urinary bacteria and the formation of other stone compositions as well. In epidemiological studies, there is a strong association between culture proven UTIs and

stone disease, with 28% of patients with stone disease having a history of UTIs[2]. In the pediatric population, an association between recurrent UTIs and idiopathic hypercalciuria has been established[3-6]. A number of studies have also investigated the impact of the intestinal microbiota on urinary stone disease; these have demonstrated that the gut microbiota may be different in stone forming patients, and that the presence of *O. formigenes* may reduce stone formation through the degradation of intestinal oxalate[7,8].

In addition, bacteria have been identified from stones through both standard culture and 16S rRNA gene sequencing methods[9,10]. Clinical cultures for bacteria were positive in 44% of pure oxalate stones, with *E. coli* being the most common organism isolated[9,10]. It is suspected, that these results grossly underestimate the presence of bacteria in stones given that standard culture protocols are not conducive to the growth of more fastidious bacteria, and that further utilization of 16S rRNA gene sequencing of bacterial DNA will show a much higher prevalence of bacteria within urinary stones[10]. Additionally, *E. coli* has been shown to promote the aggregation and growth of CaOx crystals[11,12]. The exact relationship between urinary bacteria and stone disease has yet to be determined; however, it has been postulated that bacteria may alter the host inflammatory response within the kidney thereby potentiating stone formation[13].

2.1.2 Antibiotics and Urolithiasis

Several epidemiological studies have correlated urolithiasis with antibiotic exposure in humans. Multiple studies have demonstrated an association between high rates of

antibiotic prescriptions and use, with the increased prevalence of stone disease in the United States[14-19]. Furthermore, a recent large study examining over 13 million patients in the United Kingdom revealed a significantly increased risk of the development of urolithiasis following exposure to a number of antibiotic classes including sulfa, cephalosporin, fluoroquinolone, nitrofurantoin, and penicillin[20]. The increased risk following exposure to sulfa and fluoroquinolone was especially striking with odds ratios of 2.37 and 1.84 respectively[19]. In addition, they also observed a higher risk of stone disease with more recent antibiotic exposure and exposure at younger ages[20]

It has been postulated that antibiotic use results in disruption of the microbiota and subsequent metabolic dysfunction and stone formation. This is supported by previous investigation which has demonstrated that antibiotic therapy for both cystic fibrosis and *H. pylori* infection resulted in decreased rates of *O. formigenes* colonization and higher urinary oxalate levels[21,22]. The negative impact of antibiotic exposure on urinary stone risk is further supported by a recent study which observed a four-time higher risk of urolithiasis in children with asthma, a condition associated with frequent childhood antibiotic exposure[23]. Furthermore, a mouse study which modeled pediatric antibiotic exposure demonstrated an altered host microbiota, decreased oxalate metabolism genes and fecal oxalate levels; further supporting that antibiotic exposure may potentiate stone formation through alteration of the microbiota[24].

2.2 Methods

This study did not require ethics approval from the University of Western Ontario's animal use subcommittee as the experimental use of the invertebrate DM flies are currently exempt.

2.2.1 *Drosophila melanogaster* Stone Model

2.2.1.1 Fly Husbandry

Canton-S wildtype flies (Stock #64349) were utilized for all experiments and were sourced from Bloomington Stock Center, Indiana, USE (hht://fly.bio.indiana.edu). All fly stocks were housed in wide fly vials within pre-divided trays (Genesee Scientific Corporation, CA). All DM flies were maintained in a dedicated *Drosophila* incubator (DigiTherm® *Drosophila* Incubator, Tritech Research Inc.) under standard conditions at 25°C with 40% humidity and a 12/12hr light/dark cycle. All fly stocks were inspected for signs of bacterial, fungal and mite infestation on a daily basis. Stocks were transferred to fresh food media every 3-4 days or earlier based on experiment timing in order to prevent contamination, allow for an adequate supply of media, and reduce the presence of larvae.

2.2.1.2 Preparation of Food Media

Food media was prepared on a monthly basis in order to avoid contamination and ensure appropriate freshness. The media was prepared manually in 1-liter batches. All the media ingredients were weighed with a digital scale (Sartorius AG, Goettingen, Germany), mixed with distilled water and heated into solution with a standard laboratory hot plate (Salton Appliances Corp., Dollard Des Ormeaux, Canada). The prepared media was then aliquoted into wide polypropylene fly vials (GEN32-121, Genesee Scientific, California, USA) and cooled for 8-12 hours at room temperature covered with grade 50 cheesecloth (GEN53-100, Geness Scientific, California, USA) in order to allow for adequate solidification of the media. The vials were then closed with dense cellulose acetate plugs (49-101 Diamed Inc., Mississauga, Canada) and stored at 4°C in a humidity controlled cold room. Food media was discarded after a period of one month if it was unused.

2.2.1.2.1 Standard Food Media

Standard food media was prepared according to the standard Bloomington stock center recipe (BDSC Cornmeal Food, Bloomington Stock Centre, Indiana IN). This media formulation results in firm media which resists liquefaction from larval burrowing. The ingredients used to prepare a 1-liter batch of food are listed in table 3.

Table 3: Ingredients for preparation of standard *Drosophila melanogaster* food media.

Ingredient	Product Number	Amount
Distilled water	-	1000 ml
Drosophila agar	66-103 Diamed Inc.	5.7 g
Inactive yeast	62-106 Diamed Inc.	19.3 g
Soy flour	62-115 Diamed Inc.	10 g
Yellow corn meal	62-100 Diamed Inc.	73 g
Light corn syrup	62-117 Diamed Inc.	75 ml
Propionic acid	1368 Sigma Inc.	1.25 ml
Phosphoric acid	290017 Sigma Inc.	3.75 ml

To prepare the media, 700ml distilled water was boiled and the agar was added in small amounts until completely dissolved. Proper cooking of the agar allowed for improved solidification of the food media within the fly vials. Following this, the yeast, soy flour, and corn meal were added to the solution and simmered for 10 minutes at medium heat. The remaining 300 ml of distilled water was mixed with the corn syrup and added to the solution where it was simmered for an additional 5 minutes. The acid mix was the added to inhibit bacterial and fungal growth. The solution was then cooled to 60°C and dispensed to the fly vials.

2.2.1.2.2 Lithogenic and Antibiotic Food Media

To induce CaOx stone formation within the DM flies a lithogenic diet containing 0.1% *w/v* sodium oxalate (NaOx; 71800 Sigma Inc., Oakville, Canada) was utilized. Previous analysis with scanning electron microscopy and energy dispersive x-ray spectroscopy has demonstrated that a lithogenic diet with NaOx results in the formation of COM (whewellite) and COD (weddellite) crystals similar to those observed in human urinary stone disease[25].

Administration of sub-inhibitory antibiotic treatment to the DM flies also occurred through the addition of the antibiotic to the food media. Ciprofloxacin (17850 Sigma Inc., Oakville, Canada) or TMP-SMX (A2487 Sigma Inc., Oakville, Canada) was added

to either the standard or lithogenic media at a concentration of 0.2 µg/mL or 30/10 µg/mL respectively. All agents were thoroughly dissolved in distilled water prior to being added to the food media recipe described above.

2.2.1.3 Inoculation with *Escherichia coli*

A non-urease producing strain of *E. coli* UTI89 (*E. coli* Genetic Stock Center, New Haven, United States) isolated from a human UTI was utilized to inoculate wild-type Canton-S DM flies. A single colony of UTI89 was used to inoculate 25ml of lysogeny broth (LB, 10855001, Fisher Scientific Inc., Ottawa, Canada) in a 50ml conical falcon tube (14-432-22, Fisher Scientific Inc., Ottawa, Canada) and incubated for 12 hours at 37°C. Following this the UTI89 was pelleted by centrifuging the inoculated LB at 5000 rpm for 5 minutes and the supernatant was decanted. The UTI89 pellet was then reconstituted in 1ml of 5% sucrose solution. 10µl aliquots of the UTI89 solution were then placed on top of the DM media, spread evenly over the surface with a sterile cell scraper (179693, Fisher Scientific Inc., Ottawa, Canada), and allowed to dry. Control groups were treated with 10µl of 5% sucrose solution without UTI89 using the same technique. Fresh UTI89 or control 5% sucrose was added to the media daily.

Inoculation with UTI89 was confirmed by culturing sterilized DM flies following exposure to the bacteria. DM flies were exposed to UTI89 as described above for 24 hours and then transferred to standard food media without bacteria. Flies were then

euthanized every 24 hours with carbon dioxide (CO_2) for a period of three days. The external surface of the fly was sterilized with 70% ethanol (Sigma Inc., Oakville, Canada). The DM fly was then pulverized in 50µl of phosphate buffered solution (PBS) with 0.5mm glass beads (Z250465, Sigma Inc., Oakville, Canada) utilizing the mini bead beater mill (RK-36270-02, Cole-Parmer, Montreal, Canada). 10µl of the pulverized DM fly solution was plated on an LB agar plate (2270025, Fisher Scientific Inc., Ottawa, Canada) and incubated for 12 hours at 37°C. Colony counts per fly were then determined per standard methods. *Escherichia coli* UTI89 was cultured at a minimum concentration of $3x10^3$ colony forming unit (CFU) per fly for up to three days following the 24-hour exposure with UTI89. Control flies did not demonstrate any bacterial growth and no other strains of bacteria were cultured from the DM flies.

2.2.1.4 Age and Sex Matching

In order to ensure that all adult flies utilized in all the experiments were of the same age and health, wild type Canon-S were age and sexed matched. Approximately 100-150 wild-type Canton-S DM flies were isolated in a 6oz square bottom bottle (GEN32-130, Diamed Inc., Mississauga, Canada) with standard food media along with a 1cm wide aliquot of active yeast paste (62-103 Diamed Inc., Mississauga, Canada) and grape juice agar. The active yeast and grape juice agar stimulated mating and provided additional nutrition. Mating and egg deposition occurred for 24 hours and following this the adult flies were removed. The eggs were then allowed to eclose and age matched adult flies

were collected after 4 days and sorted according to gender. The flies were sorted by gender following CO_2 narcotization on the fly pad (Flystuff Inc., San Diego, United States). The age and sex matched adult flies were then utilized in all the experiments.

2.2.1.4.1 Preparation of Grape-Juice Agar Plates

Grape-juice agar was prepared in 500ml batches on a monthly basis using the ingredients listed in table 4. The agar and sucrose were added to the distilled water and heated until completely dissolved. The grape-juice concentrate was the added and thoroughly mixed. The solution was then cooled to 60°C and the ethanol and glacial acetic acid was added in order to inhibit bacterial and fungal growth. The solution was then aliquoted into 100mm plastic petri dishes (VWR Inc., Radnor, United States) and solidified at room temperature. The plates were stored at 4°C in a humidity controlled cold room and discarded after a month if unused.

Table 4: Ingredients for preparation of grape-juice agar plates.

Ingredient	Product Number	Amount
Distilled water	-	375 ml
Bacteriological agar	5306 Sigma Inc.	12 g
Sucrose	7903 Sigma Inc.	22 g
Grape juice	Welch's grape-juice concentrate	110 ml
Ethanol	Sigma Inc.	10 ml
Glacial acetic acid	2183 Sigma Inc.	5 ml

2.2.1.5 Lifespan Studies

Fifty age and sex matched wild type Canton-S flies were anesthetized with CO_2 narcotization and added to either standard media or lithogenic food media with or without *E. coli* UTI89. Three separate replicates were performed for every treatment group. UTI89 or sucrose control was added to the media daily as described above. Fly media was exchanged every 3 days to avoid competition for fresh food which could potentially impact survival. DM fly deaths were recorded daily, and any flies lost during transfer were censored. The adult flies were followed until the last death.

2.2.1.6 Calcium Oxalate Stone Formation in *Drosophila melanogaster* Following Exposure to *Escherichia coli* and Antibiotics

Thirty age and sex matched wild type Canton-S flies were added to wide vials containing either standard or lithogenic food media with or without *E. coli* UTI89 exposure for either a 7- or 14-day assay. Treatment with ciprofloxacin (0.2 μg/mL) or TMP-SMX (30/10 μg/mL) occurred on day 5-7 of the assay. Following completion of the assay the DM flies were removed, euthanized and processed for imaging. Three separate replicates were performed for each of the different treatment groups and assay timelines. A schematic of the various treatment groups is presented in figure 3.

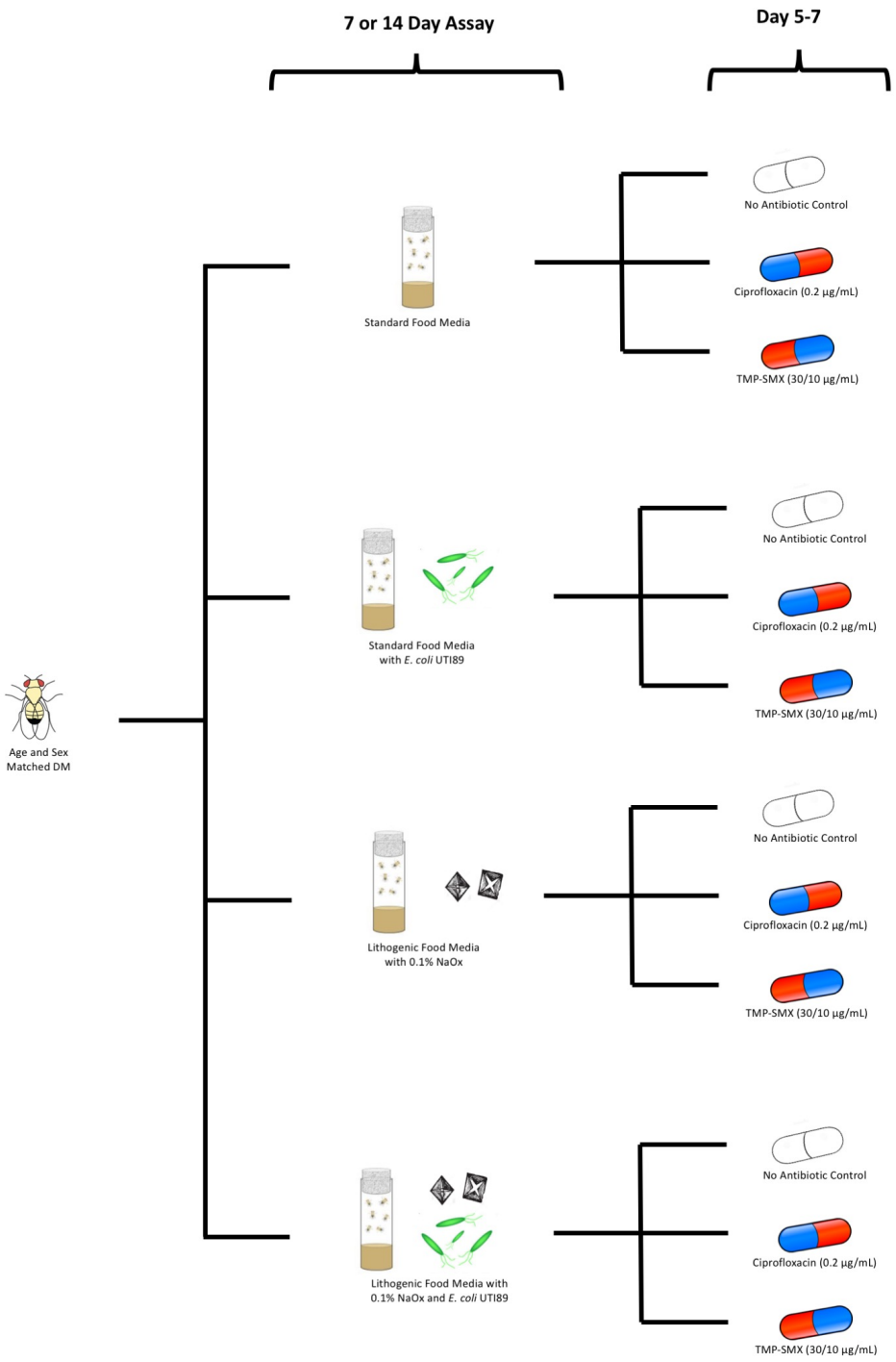

Figure 3: Schematic representation of treatment groups in *Drosophila melanogaster* fly model.

2.2.1.6.1 Dissection of *Drosophila melanogaster* Malpighian Tubules

Malpighian tubules were dissected from DM flies immediately following euthanization with CO_2. Dissections were performed under a dissecting light microscope (AmScope Inc., Irvine, United States) on a culture dish in Schneider's *Drosophila* medium (0146 Sigma Aldrich Inc., Oakville, Canada) utilizing fine forceps (Fine Science Tools Inc., Vancouver, Canada). Schneider's Media was selected as a dissection medium as its composition most closely resembles insect hemolymph and prevents osmotic cell lysis. Dissections were carried out in a Sylgard lined Pyrex Petri dish (3160100 Sigma Aldrich Inc., Oakville, Canada) to provide additional protection to the delicate DM tissues. A minimal touch technique was utilized where the DM fly was anchored with forceps at the superior aspect of the thorax. The anal region or Terminalia of the DM fly was then grasped with forceps and the hindgut was removed with gentle pressure. Following the hindgut, the anterior and posterior tubules emerged. The midgut was then severed, and the fly was discarded.

The dissected Malpighian tubules were then fixed in 4% formaldehyde (252549, 0146 Sigma Aldrich Inc., Oakville, Canada) in PBS for 1 hour and mounted on a frosted microscope slide (510D, Fisher Scientific Inc., Ottawa, Canada) in 50% glycerol (G5516, 0146 Sigma Aldrich Inc., Oakville, Canada) in PBS with a 22mm glass coverslip (3400, Fisher Scientific Inc., Ottawa, Canada). The edges of the coverslip were sealed with clear nail polish. Birefringent microscopy images of CaOx crystals within dissected

Malpighian tubules were obtained with an inverted light microscope (Nikon Inc., Tokyo, Japan) with a 100x magnification.

2.2.2 Calcium Oxalate Crystal Adherence

2.2.2.1 Cell Culture

Madin-Darby Canine Kidney (MDCK) renal epithelial cells (ATCC-CCL-34, NBL-2, Manassas, VA) were utilized for the crystal adherence assay. A stock of MDCK cells was cultured in T75 cell culture flasks (156499, Fisher Scientific Inc., Ottawa, Canada) at 37°C in 5% CO_2 utilizing a Forma Steri-Cycle CO_2 incubator (370, Fisher Scientific Inc., Ottawa, Canada). The MDCK cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA). No antibiotic additives were included in the cell media. The MDCK cells were cultured to 90% confluence and then passaged onto 150 mm cell culture plates (172931, Thermo Fisher Scientific, Waltham, MA) at a concentration of $5.0x10^6$ cells per plate. In order to passage the cells onto the cell culture plates, the adherent cells were incubated with 0.5% trypsin (15400054, Thermo Fisher Scientific, Waltham, MA) for 10 minutes to allow for cell detachment. The detached cells were the centrifuged at 1000 rpm for 5 minutes at 37°C in a 15 ml conical falcon tube (T1943, Sigma Aldrich Inc., Oakville, Canada). The

cell pellet was the resuspended in 2 ml of cell media and cell concentration was determined with a hemocytometer. Cell media was exchanged every three days and cells were inspected under the microscope to ensure viability. MDCK cells were used until a maximum passage of ten and then exchanged for a fresh stock. Once cells reached 90% confluence on the 150mm cell culture dishes they were utilized for the crystal adherence assay.

2.2.2.2 Crystal Adherence Assay

In order to conduct the crystal adherence assay, MDCK cells were grown to 90% confluence in 150mm cell culture dishes. The MDCK cells were then exposed to *E. coli* UTI89 at a concentration of 10^3 CFU suspended in standard cell culture media for 20 minutes at 37°C. The UTI89 was then removed by gently washing the MDCK cells with 5ml of fresh cell culture media twice. Following this, the MDCK cells were incubated with CaOx crystal suspension (0.5 mg/mL; 455997, Sigma Aldrich Inc., Oakville, Canada) in artificial urine with or without the addition of ciprofloxacin (0.2 μg/mL) or TMP-SMX (30/10 μg/mL) for an additional 20 minutes. The artificial urine was prepared with the ingredients listed in table 5 according to the protocol previously described[26]. The properties of the artificial urine are listed in table 6. Once again, the MDCK cells were gently washed with cell culture media twice in order to remove any unattached crystals. Fresh cell culture media was then replaced, and the cells were immediately imaged with birefringent microscopy utilizing an inverted light microscope

(Nikon Inc., Tokyo, Japan) under a 100x magnification prior to cell death occurring. Five separate cell culture dishes per treatment group were utilized for the experiment, and three separate replicates of all treatment groups were performed.

Table 5: Ingredients for the preparation of artificial urine[26].

Ingredient	Amount
Urea	2.427 g
Uric acid	0.034 g
Creatinine	0.090 g
$Na_3C_6H_5O_7 \cdot 2H_2O$	0.297 g
NaCl	0.634 g
KCl	0.450 g
NH_4Cl	0.161 g
$CaCl_2 \cdot 2H_2O$	0.089 g
$MgSO_4 \cdot 7H_2O$	0.100 g
$NaHCO_3$	0.034 g
NaC_2O_4	0.003 g
Na_2SO_4	0.258 g
$NaH_2PO_4 \cdot H_2O$	0.100
Na_2HPO_4	0.011 g
Deionized water	200 ml

Table 6: Properties and compositions of artificial urine[26].

Property and Composition	Concentration
pH	6.2
Specific gravity	1.010 g/ml
Osmolality	446 mOsm/kg
Urea	200 mM
Uric acid	1.00 mM
Creatinine	4.00 mM
$Na_3C_6H_5O_7$	5.00 mM
NaCl	54.00 mM
KCl	30.00 mM
NH_4Cl	15.00 mM
$CaCl_2$	3.00 mM
$MgSO_4$	2.00 mM
$NaHCO_3$	2.00 mM
NaC_2O_4	0.10 mM
Na_2SO_4	9.00 mM
NaH_2PO_4	3.60 mM
$NaHPO_4$	0.40 mM

2.2.3 Data Analysis

Pixel intensity of birefringent CaOx crystals were quantified utilizing MATLAB (MathWorks, 2018) using a threshold pixel intensity of 150. For processing of the dissected Malpighian tubules only the tubules of interest were outlined and included within the analysis. Background pixel intensity was subtracted for all image analysis. Statistical analysis was performed with SPSS version 25.0 (IBM, New York, USA) using a one-way analysis of variance (ANOVA) with a p value of <0.05. For survival curve analysis the log-rank test was used to determine the significance of change.

2.3 Results

2.3.1 *Drosophila melanogaster* Model

2.3.1.1 *Drosophila melanogaster* Survival with Lithogenic Diet and *Escherichia coli* Exposure

Treatment of DM flies with *E.coli* did not affect fly survival, and both control and UTI89 treated flies survived for a median of 33 days on standard food media. This effect was consistent in flies fed a lithogenic diet, with the oxalate control and oxalate with UTI89 DM flies surviving a median of 23 and 24 days respectively. The mean and median survival time is summarized in table 7. However, treatment with the lithogenic diet

resulted in significantly reduced survival time compared with DM flies fed a standard diet ($p<0.0001$, table 8). A Kaplan-Meier curve comparing survival among different treatment groups is illustrated in figure 4.

Table 7: Mean and median survival of *Drosophila melanogaster* following treatment with sodium oxalate (0.1%) or *Escherichia coli* UTI89. Estimation is limited to the largest survival time if it is censored, (n=150/group).

Treatment	Mean				Median			
			95% Confidence Interval				95% Confidence Interval	
	Est.	Std. Error	Lower	Upper	Est.	Std. Error	Lower	Upper
Control	30.174	1.503	27.229	33.120	33.000	0.801	31.430	34.570
UTI89	29.807	1.584	26.701	32.912	33.000	1.226	30.597	35.403
Oxalate Control	21.461	1.162	19.184	23.737	23.000	2.098	18.888	27.112
Oxalate with UTI89	22.279	1.123	20.594	24.996	25.000	.983	25.074	28.926

Table 8: Statistical analysis of the survival distribution of *Drosophila melanogaster* following treatment with sodium oxalate (0.1%) or *Escherichia coli* UTI89, (n=150/group).

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	33.557	3	0.000
Breslow (Generalized Wilcoxon)	12.380	3	0.006
Tarone-Ware	21.446	3	0.000

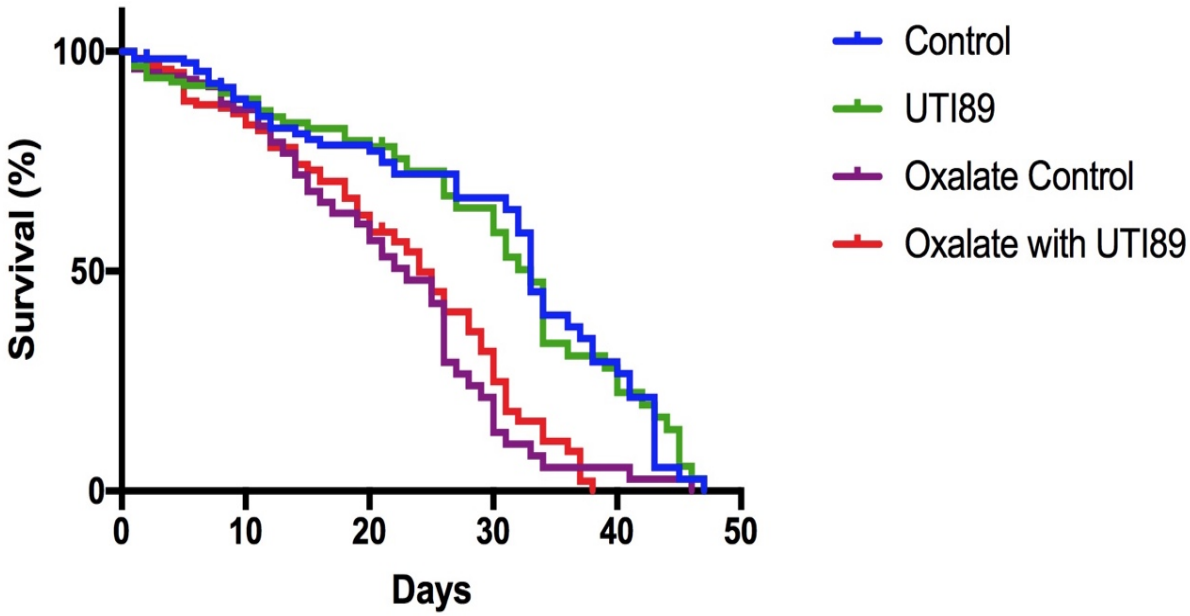

Figure 4: Kaplan Meier survival analysis of *Drosophila melanogaster* following exposure to sodium oxalate (0.1%) lithogenic diet or *Escherichia coli* UTI89 (n=150/group).

2.3.1.2 Calcium Oxalate Stone Formation in *Drosophila melanogaster* Malpighian Tubules

2.3.1.2.1 Calcium Oxalate Stone Formation in *Drosophila melanogaster* Malpighian Tubules following *Escherichia coli* Exposure: A Timed Analysis

To assess for appropriate time points for the treatment assays, wild-type Canton-S DM were exposed to either a standard or lithogenic diet with 0.1% NaOx for 21 days with or without the addition of *E. coli* UTI89. Malpighian tubules were dissected and analyzed for CaOx crystal deposition at days 7, 14, and 21 under a birefringent light microscope. DM flies fed the standard diet did not form any CaOx crystals within the dissected Malpighian tubules. Representative images of the CaOx crystals within the dissected Malpighian tubules at the different timepoints are shown in figure 5.

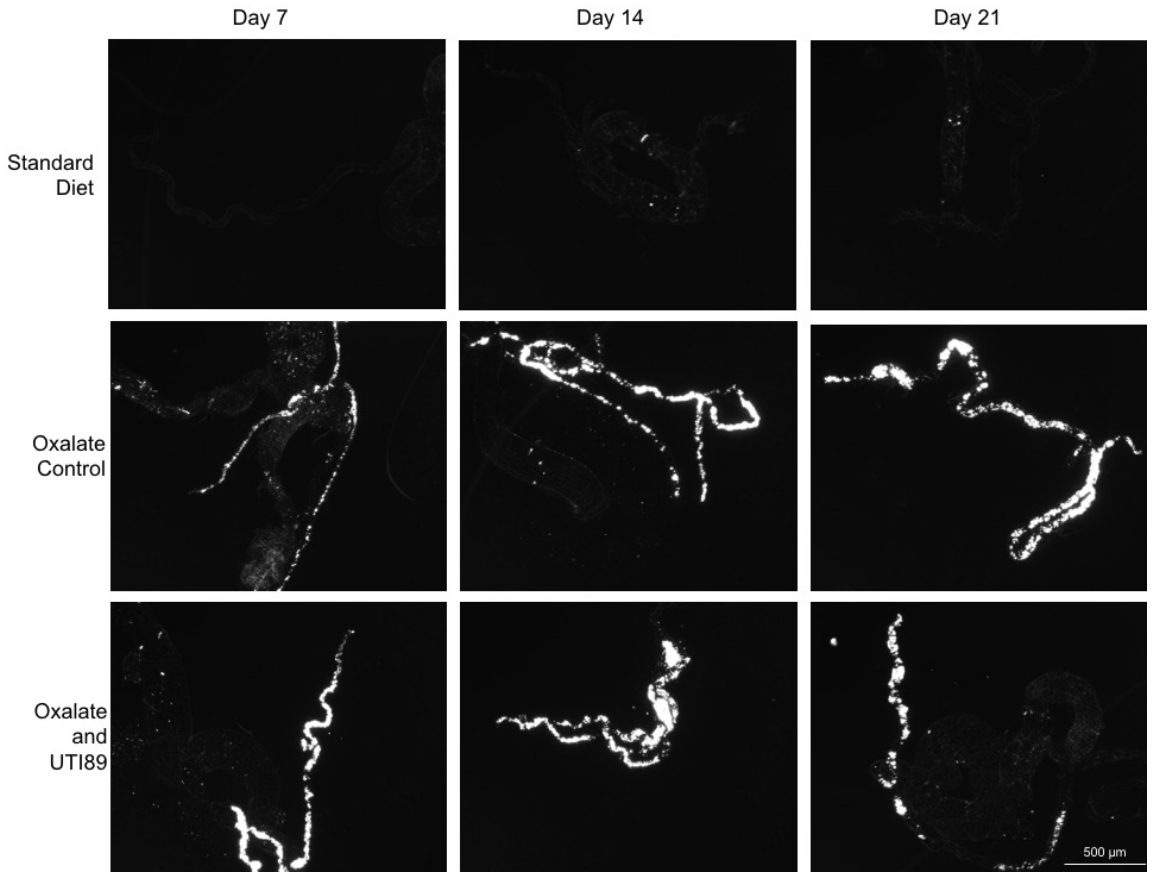

Figure 5: Calcium oxalate stone formation in dissected Malpighian tubules following exposure to standard diet of sodium oxalate (0.1%) lithogenic diet with or without *Escherichia coli* UTI89 at days 7, 14, and 21 under 100x magnification.

Comparisons between the oxalate control tubules demonstrated increasing amounts of CaOx crystal deposition with longer exposure to the lithogenic diet as would be expected. The amount of CaOx crystal deposition appeared to plateau around the 21-day time point and was consistent with the median survival time for the DM flies fed the lithogenic media. In addition, exposure of the DM flies to *E. coli* UTI89 appeared to increase CaOx crystal deposition at the day 7 and 14 timepoints; however, the difference was less striking at the later 14-day timepoint. There did not appear to be any difference in CaOx crystal deposition between the oxalate control and oxalate with UTI89 treatment group at the latest 21-day timepoint. Given this data, a 7- and 14-day assay time period was selected for the remainder of the experiments.

2.3.1.2.2 Calcium Oxalate Stone Formation in *Drosophila melanogaster* Malpighian Tubules Following *Escherichia coli* Exposure

Inoculation of DM flies with *E. coli* UTI89 was observed to significantly increase CaOx stone formation within dissected Malpighian tubules following 7 days of exposure ($p=0.005$); however, there was no difference noted in stone formation with the longer 14-day treatment period. Representative images of the CaOx crystals within the dissected Malpighian tubules are shown in figure 6. The mean measured pixel intensity of the CaOx crystals within the tubules stratified by treatment group is displayed in figure 7.

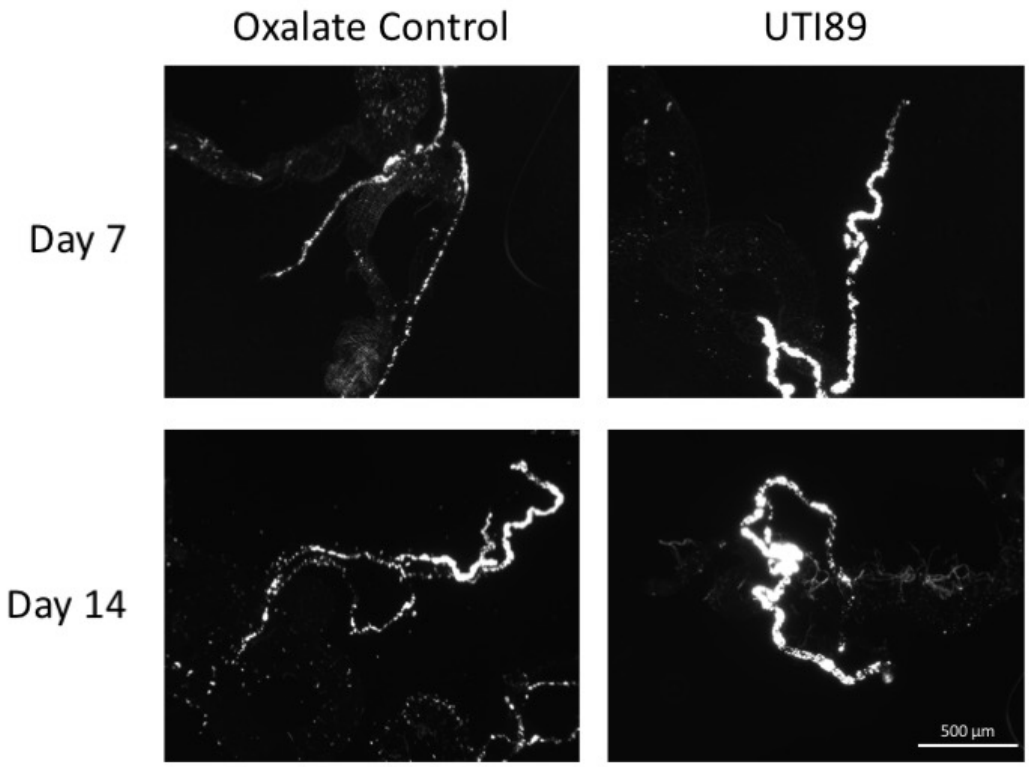

Figure 6: Representative birefringence microscopy images of calcium oxalate stone formation in dissected Malpighian tubules following exposure to sodium oxalate (0.1%) lithogenic diet with or without *Escherichia coli* UTI89 at days 7 and 14 under 100x magnification.

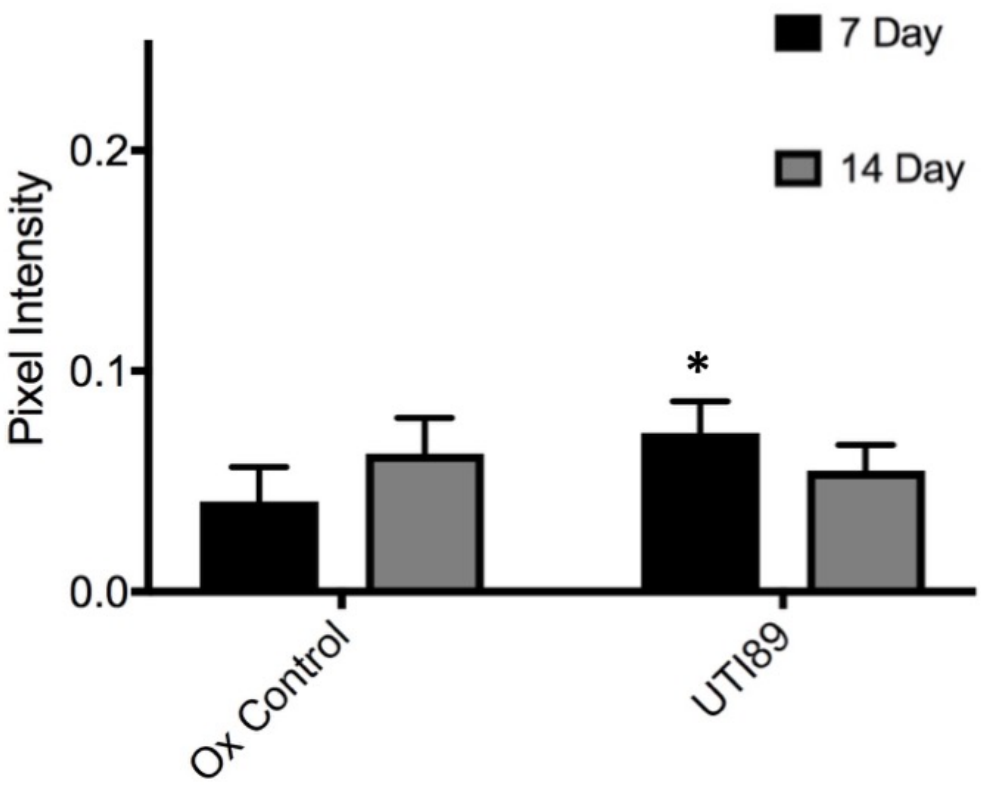

Figure 7: Measured pixel intensity of calcium oxalate stone formation in dissected Malpighian tubules following exposure to sodium oxalate (0.1%) lithogenic diet with or without *Escherichia coli* UTI89 at days 7 and 14. Data displayed as mean ± SEM (n=29-80/group), *p<0.05 compared with day 7 oxalate control.

2.3.1.2.3 Calcium Oxalate Stone Formation in *Drosophila melanogaster* Malpighian Tubules Following Antibiotic Treatment

Wild-type Canton-S DM flies were observed to have significant increases in CaOx stone burden following exposure to both ciprofloxacin (0.5μg/mL; $p<0.001$) and TMP-SMX (30/10 μg/mL; $p<0.001$) at the 7-day time point. In addition, CaOx crystal deposition was noted to further increase in both groups at the 14-day time point ($p<0.05$). This is interesting given that the actual antibiotic treatment duration and time points were the same for both the 7- and 14-day assays; with the DM flies only being exposed to antibiotics on days 5-7 of each assay. Comparatively, TMP-SMX exhibited a trend towards greater CaOx stone formation compared with ciprofloxacin; however, this was not statistically significant. Representative microscopy images of the CaOx crystals within the dissected Malpighian tubules are displayed in figure 8. The mean measured pixel intensity of the CaOx crystals within the tubules stratified by treatment group is demonstrated in figure 9.

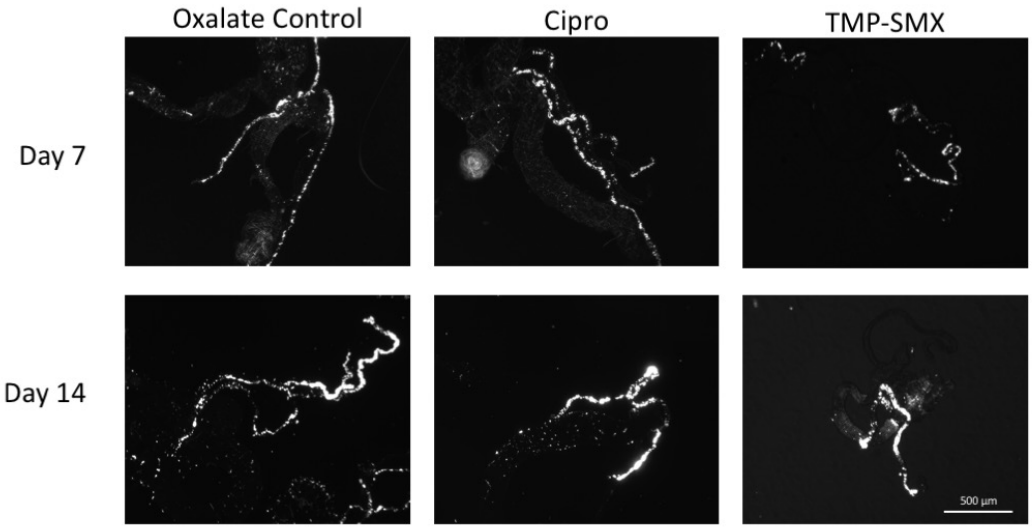

Figure 8: Representative birefringence microscopy images of calcium oxalate stone formation in dissected Malpighian tubules following exposure to sodium oxalate (0.1%) lithogenic diet with or without ciprofloxacin (0.2 μg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 μg/mL) at days 7 and 14 under 100x magnification.

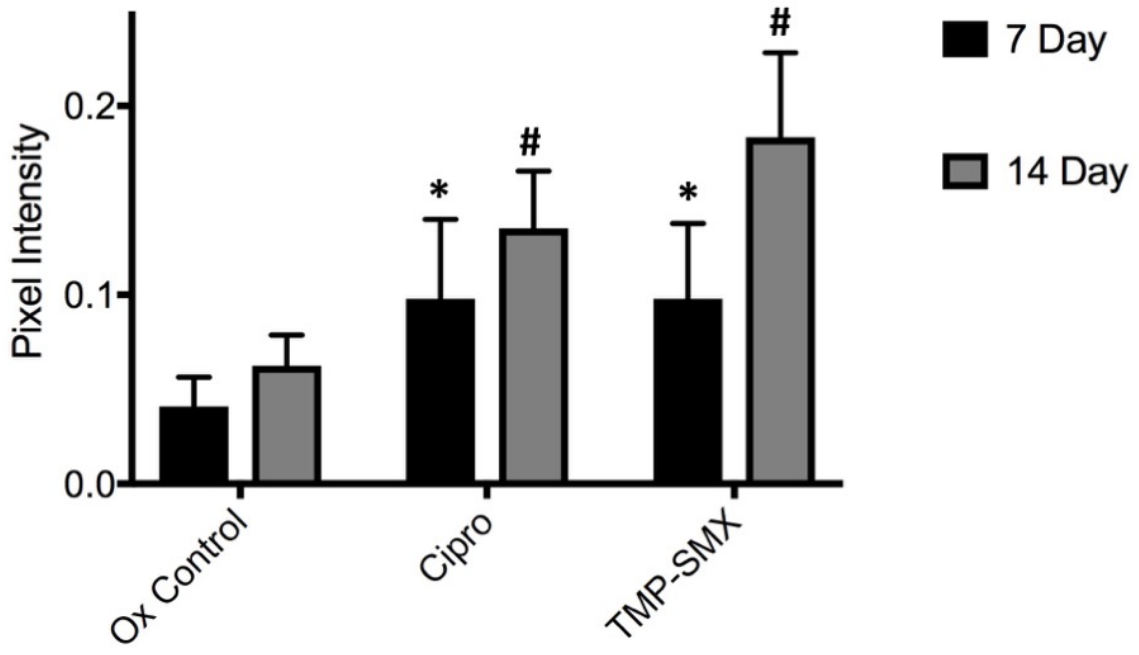

Figure 9: Measured pixel intensity of calcium oxalate stone formation in dissected Malpighian tubules following exposure to sodium oxalate (0.1%) lithogenic diet with or without ciprofloxacin (0.2 μg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 μg/mL) at days 7 and 14. Data displayed as mean ± SEM (n=29-80/group), *p<0.05 compared with day 7 oxalate control, #p<0.05 compared with day 14 oxalate control.

2.3.1.2.4 Calcium Oxalate Stone Formation in *Drosophila melanogaster* Malpighian Tubules Following Combination Treatment with *Escherichia coli* UTI89 and Antibiotics

In order to further investigate the potential interaction between urinary pathogens and antibiotic exposure, DM flies were co-treated with a combination of UTI89 and either antibiotic. It was observed that co-treatment of the DM flies with both *E. coli* UTI89 and either antibiotic had no overall effect on stone burden for both measured time points at day 7 and 14. The combination of the *E. coli* and antibiotic appeared to negate each other's effects and the overall stone burden was not observed to be any different compared to the oxalate control group. For comparison purposes representative microscopy images of the CaOx crystals within the dissected Malpighian tubules for all the treatment groups including the combination treatment are displayed in figure 10. In addition, the mean measured pixel intensity of the CaOx crystals within the tubules stratified by treatment group is shown in figure 11. Details of the ANOVA statistical analysis for all group comparisons are summarized in tables 9-11.

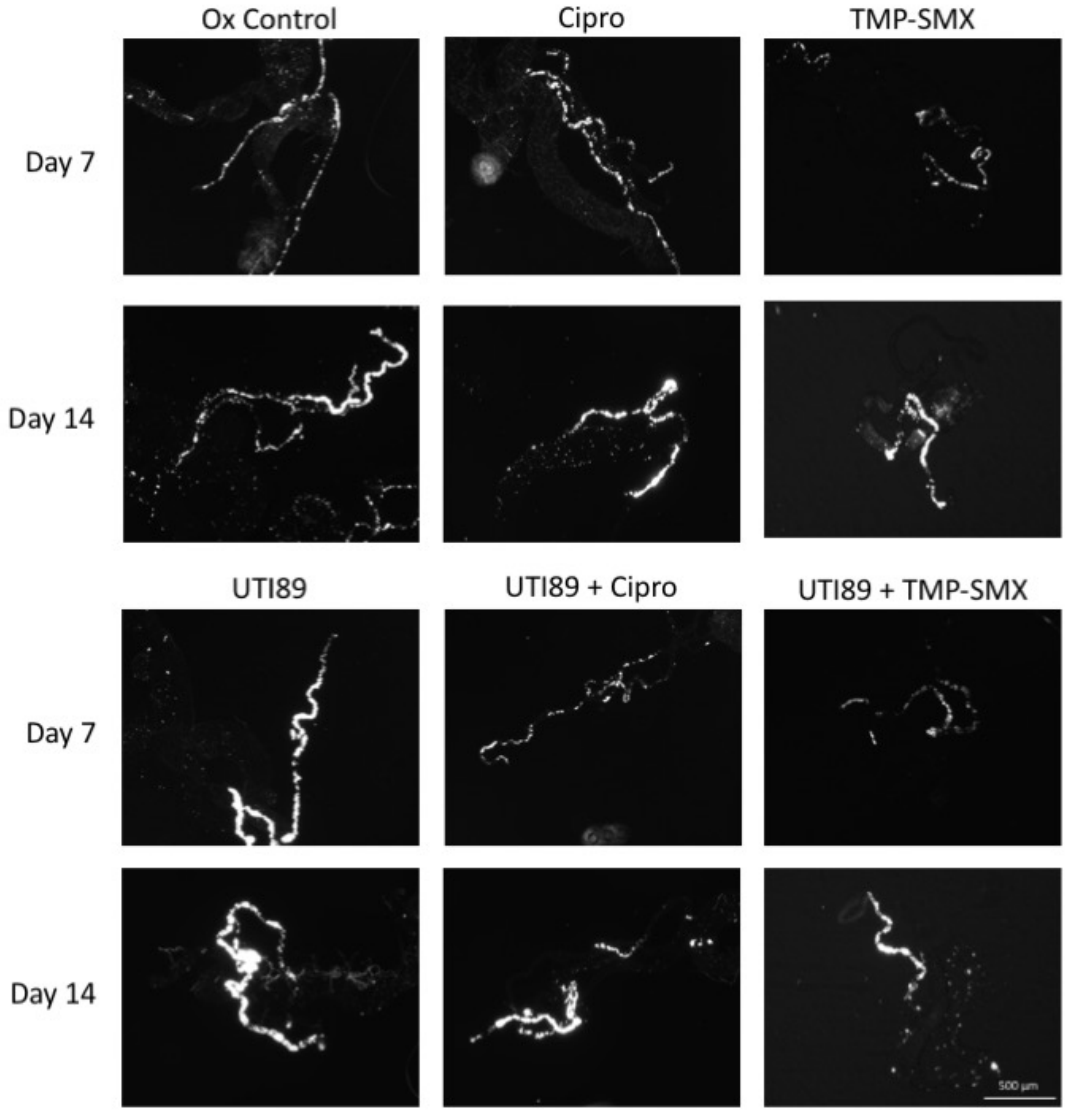

Figure 10: Representative birefringence microscopy images of calcium oxalate stone formation in dissected Malpighian tubules following exposure to sodium oxalate (0.1%) lithogenic diet with or without *Escherichia coli* UTI89, ciprofloxacin (0.2 µg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 µg/mL) at days 7 and 14 under 100x magnification.

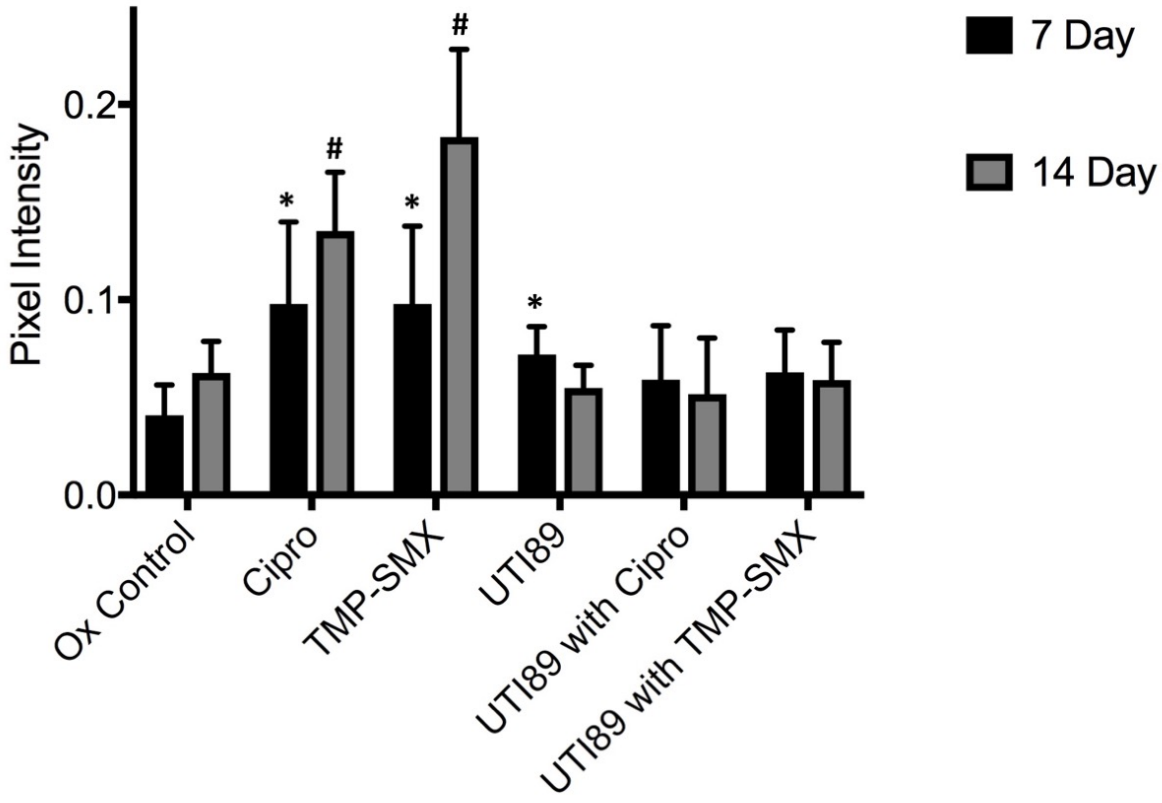

Figure 11: Measured pixel intensity of calcium oxalate stone formation in dissected Malpighian tubules following exposure to sodium oxalate (0.1%) lithogenic diet with or without *Escherichia coli* UTI89, ciprofloxacin (0.2 μg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 μg/mL) at days 7 and 14. Data displayed as mean ± SEM (n=28-80/group), * p<0.05 compared with day 7 oxalate control, #p<0.05 compared with day 14 oxalate control.

Table 9: Mean measured pixel intensity of calcium oxalate stone formation in dissected Malpighian tubules following exposure to sodium oxalate (0.1%) lithogenic diet with or without *Escherichia coli* UTI89, ciprofloxacin (0.2 μg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 μg/mL) at days 7 and 14.

					95% CI	
Day 7	**N**	**Mean**	**SD**	**Std. Error**	**Lower**	**Upper**
Oxalate Control	79	0.0410	0.06931	0.00780	0.0255	0.0566
Cipro	40	0.1100	0.13704	0.02167	0.0662	0.1539
TMP-SMX	48	0.0978	0.13873	0.2002	0.575	0.1381
UTI89	69	0.0719	0.06000	0.00722	0.0575	0.0863
UTI89 with Cipro	30	0.0554	0.07143	0.01304	0.0287	0.0821
UTI89 with TMP-SMX	51	0.0652	0.07979	0.01117	0.0428	0.0877
Day 14						
Oxalate Control	42	0.0626	0.05225	0.00806	0.0463	0.0789
Cipro	69	0.1341	0.12509	0.01506	0.1041	0.1642
TMP-SMX	68	0.1833	0.18707	0.02269	0.1380	0.2286
UTI89	28	0.0549	0.03064	0.00579	0.0431	0.0668
UTI89 with Cipro	63	0.0517	0.11503	0.01449	0.0227	0.0807
UTI89 with TMP-SMX	47	0.0590	0.07695	0.01122	0.0364	0.0816

Table 10: ANOVA analysis of mean measured pixel intensity of calcium oxalate stone formation in dissected Malpighian tubules following exposure to sodium oxalate (0.1%) lithogenic diet with or without *Escherichia coli* UTI89, ciprofloxacin (0.2 μg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 μg/mL) at days 7 and 14.

	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	1.215	11	0.110	9.337	0.000
Within Groups	7.361	622	0.012		
Total	8.577	633			

Table 11: ANOVA analysis with Tukey post-hoc analysis for multiple group comparison of mean measured pixel intensity of calcium oxalate stone formation in dissected Malpighian tubules following exposure to sodium oxalate (0.1%) lithogenic diet with or without *Escherichia coli* UTI89, ciprofloxacin (0.2 μg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 μg/mL) at days 7 and 14.

Group Comparison		Mean Difference	Std. Error	Sig.	95% CI Lower	95% CI Upper
Day 7						
Oxalate Control						
	Cipro	-0.09311	0.01793	0.000	-0.1519	-0.0343
	TMP-SMX	-0.14227	0.01800	0.000	-0.2013	-0.0832
	UTI89	-0.6901	0.02111	0.005	-0.1383	0.0002
	UTI89 with Cipro	-0.01436	0.02333	1.000	-0.0710	0.0496
	UTI89 with TMP-SMX	-0.02421	0.01954	0.986	-0.0883	0.00399
Day 14						
Oxalate Control						
	Cipro	-0.07156	0.02129	0.039	-0.1414	-0.0017
	TMP-SMX	-0.12072	0.02135	0.000	-0.1908	-0.0507
	UTI89	0.00765	0.02654	1.000	-0.0792	0.0605
	UTI89 with Cipro	0.01089	0.02167	1.000	-0.0602	0.0820
	UTI89 with TMP-SMX	0.00362	0.02310	1.000	-0.0722	0.0794

2.3.2 Calcium Oxalate Crystal Adherence Assay

2.3.3 Calcium Oxalate Crystal Adherence to Renal Epithelial Cells Following *Escherichia coli* Exposure

To further evaluate the potential mechanism that urinary pathogens may alter stone formation, renal epithelial cells were exposed to a non-urease producing strain of *E. coli* and adherence of CaOx crystals was measured. Exposure of MDCK renal epithelial cells to *E. coli* UTI89 was observed to result in a significant increase in CaOx crystal adherence compared to control ($p=0.001$). This correlated with the results observed in the DM fly model where UTI89 exposure was also observed to increase CaOx stone burden. Representative birefringent microscopy images of the CaOx crystals adhered to the MDCK cells are displayed in figure 12. The mean measured pixel intensity of the CaOx crystals stratified by treatment group is displayed in graphical form in figure 13.

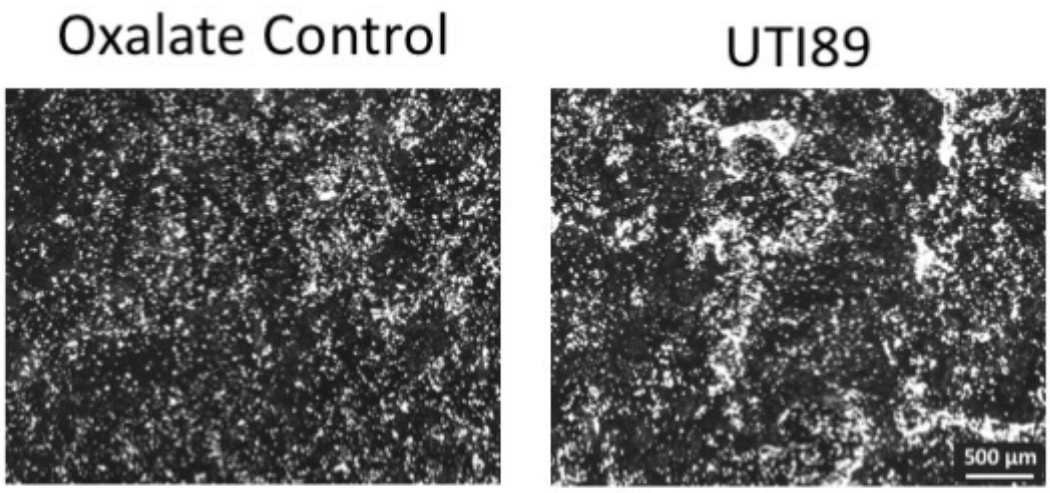

Figure 12: Representative birefringence microscopy images of calcium oxalate crystal adherence to MDCK renal epithelial cells following exposure to *Escherichia coli* UTI89 (10^3 CFU) under 100x magnification.

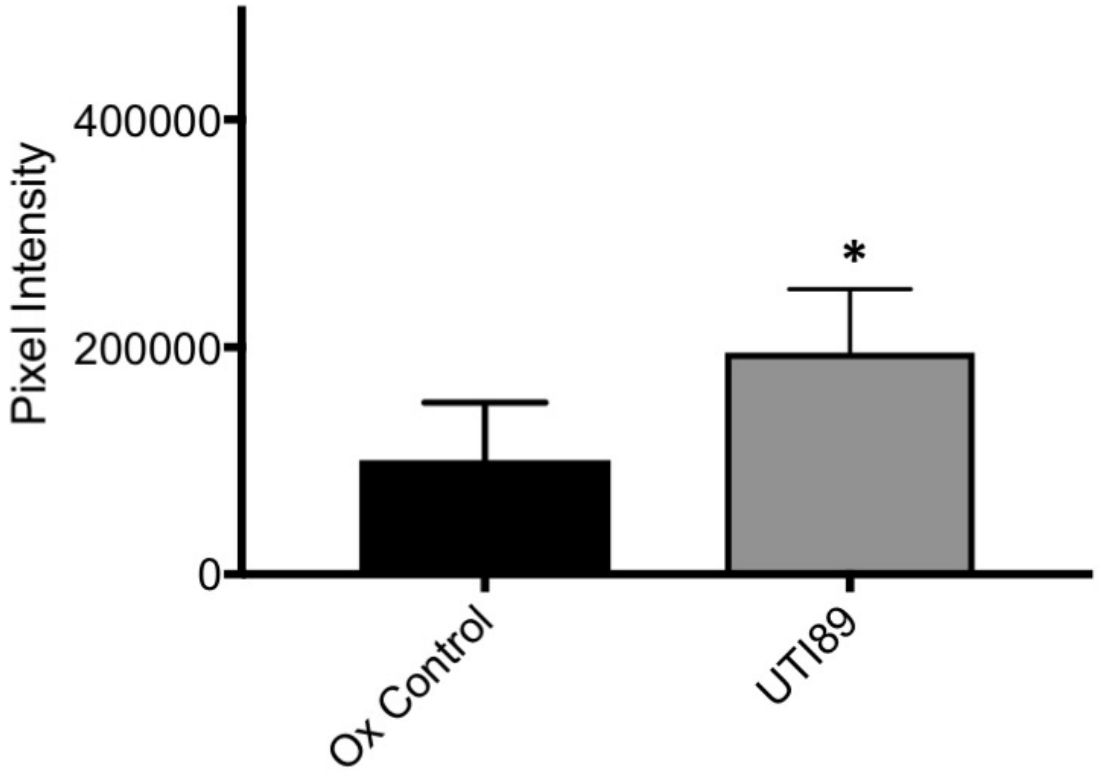

Figure 13: Measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells following exposure to *Escherichia coli* UTI89 (10^3 CFU). Data displayed as mean ± SEM (n=15/group), * $p<0.05$ compared with control.

2.3.3.1 Calcium Oxalate Crystal Adherence to Renal Epithelial Cells Following Treatment with Antibiotics

Exposure of the MDCK renal epithelial cells to the two different antibiotic classes had differing treatment effects. Treatment of MDCK cells with ciprofloxacin was observed to increase CaOx crystal adhesion ($p<0.001$). Whereas, treatment with TMP-SMX did not appear to significantly alter crystal adhesion compared to the control. This is in contrast to the results observed in the DM fly model where both antibiotic classes were observed to significantly increase stone formation within the Malpighian tubules. Representative microscopy images of CaOx crystal adherence to the MDCK cells are shown in figure 14 and the mean measured pixel intensity of the CaOx crystals stratified by treatment group is displayed in figure 15.

Figure 14: Representative birefringence microscopy images of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with ciprofloxacin (0.2 μg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 μg/mL) under 100x magnification.

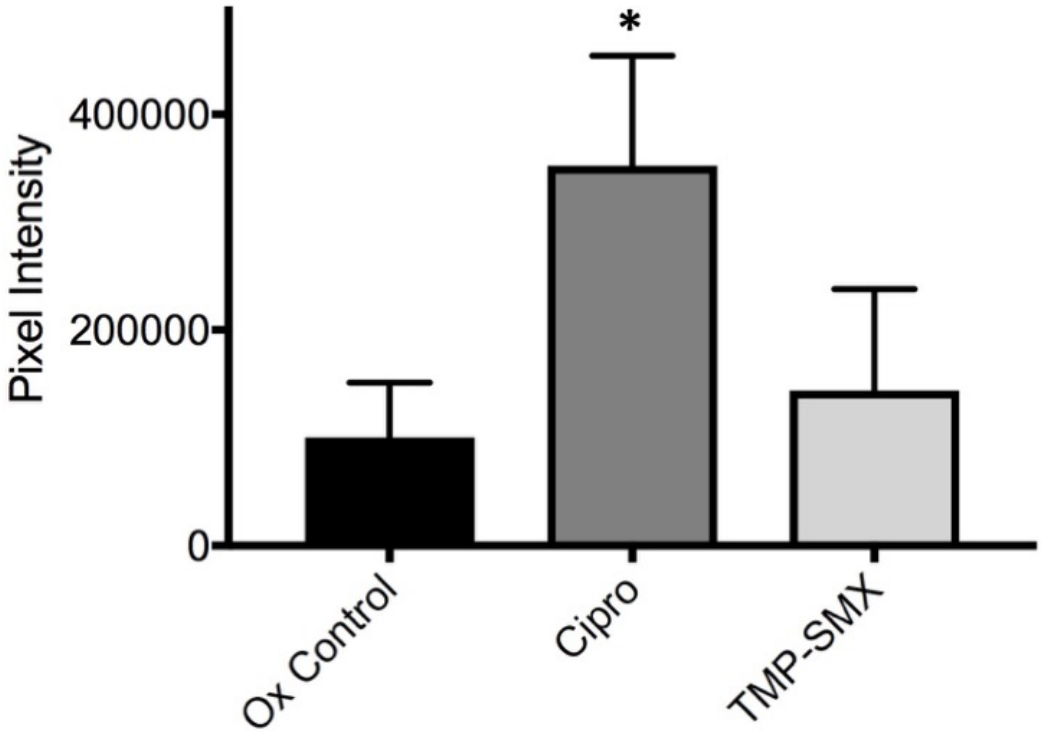

Figure 15: Measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells treated with ciprofloxacin (0.2 μg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 μg/mL). Data displayed as mean ± SEM (n=15/group), * p<0.05 compared with control.

2.3.3.2 Calcium Oxalate Crystal Adherence to Renal Epithelial Cells Following Combination Treatment with *Escherichia coli* and Antibiotics

Once again, in order to further evaluate the potential interaction between urinary pathogens and antibiotic exposure, MDCK cells were exposed to a combination of UTI89 and either antibiotic treatment. MDCK cells treated with the combination of UTI89 and either antibiotic were shown to have no difference in adherence of CaOx crystals compared to the control. This was very similar to the results observed in the DM fly model were the combination of urinary pathogen exposure and antibiotic treatment appeared to negate each other's effect and result in no overall difference in stone burden. For comparison purposes representative microscopy images of CaOx crystal adherence to MDCK cells for all the treatment groups including the combination treatment are displayed in figure 16. In addition, the mean measured pixel intensity of the CaOx crystals stratified by treatment group is shown in figure 17. Details of the ANOVA statistical analysis for all group comparisons are summarized in tables 12-14.

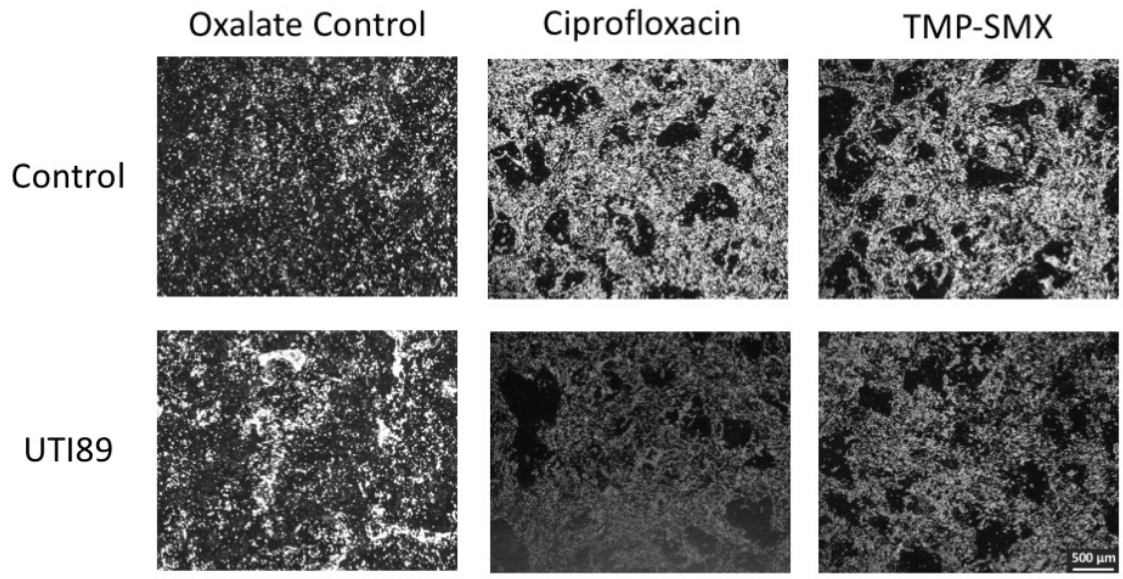

Figure 16: Representative birefringence microscopy images of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with or without *Escherichia coli* UTI89 (10^3 CFU), ciprofloxacin (0.2 μg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 μg/mL) under 100x magnification.

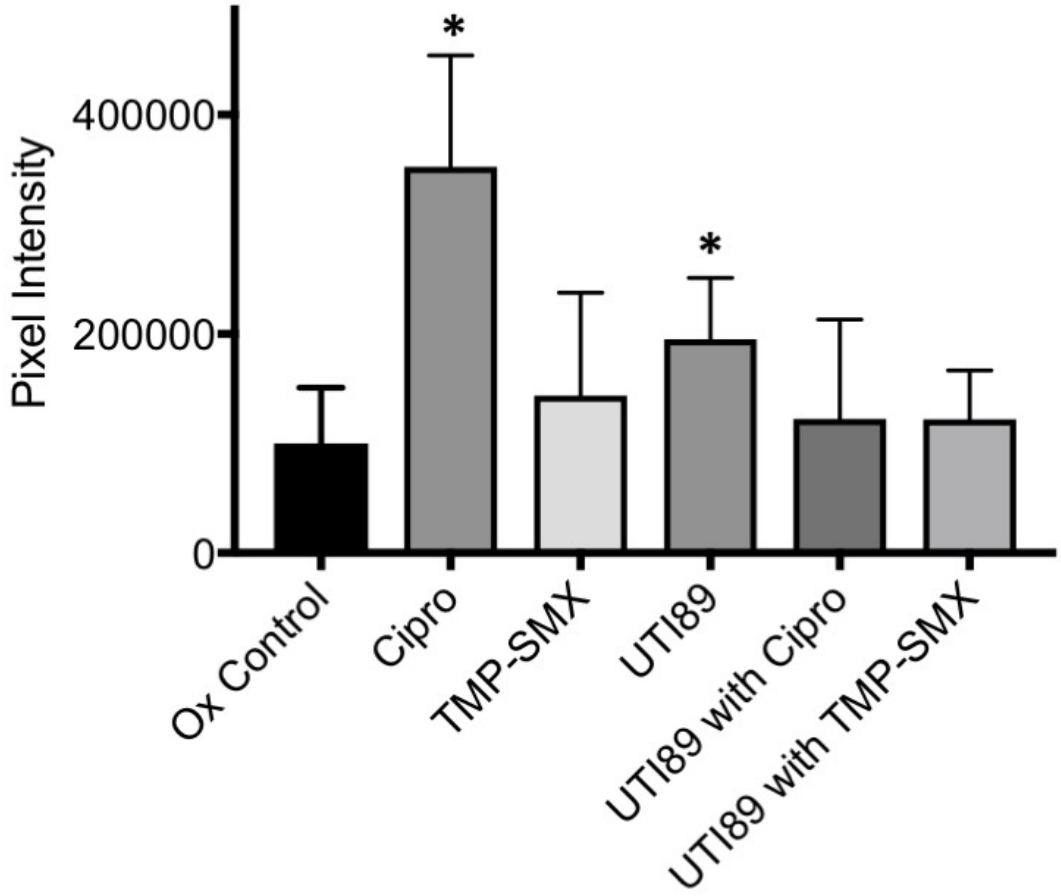

Figure 17: Measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with or without *Escherichia coli* UTI89 (10^3 CFU), ciprofloxacin (0.2 μg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 μg/mL). Data displayed as mean ± SEM (n=15/group), * $p<0.05$ compared with control.

Table 12: Mean measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with or without *Escherichia coli* UTI89, ciprofloxacin (0.2 μg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 μg/mL).

					95% CI	
	N	Mean	SD	Std. Error	Lower	Upper
Oxalate Control	15	86039.9	46463.8	11996.9	60309.1	30932.0
Cipro	15	352201.8	94654.6	24439.7	299783.8	404619.8
TMP-SMX	15	144028.6	86535.8	22343.4	96106.7	191950.5
UTI89	15	195313.2	51780.0	13369.5	166638.4	223988.1
UTI89 with Cipro	15	122517.0	83892.1	21660.8	76059.1	168974.9
UTI89 with TMP-SMX	15	123983.9	40721.6	10514.3	101433.0	146534.7

Table 13: ANOVA analysis of mean measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with or without *Escherichia coli* UTI89, ciprofloxacin (0.2 μg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 μg/mL).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.890E+11	5	1.378E+11	27.573	0.000
Within Groups	4.198E+11	84	4.997E+9		
Total	1.109E+12	89			

Table 14: ANOVA analysis with Tukey post-hoc analysis for multiple group comparison of mean measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with or without *Escherichia coli* UTI89, ciprofloxacin (0.2 μg/mL) or trimethoprim-sulfamethoxazole (TMP-SMX; 30/10 μg/mL).

Group		Mean Difference	Std.	Sig.	95% CI Lower	95% CI Upper
Oxalate						
	Cipro	-266161.9	25813.05	0.000	-341446.8	190877.0
	TMP-SMX	-57988.7	85601.0	.228	-133273.6	17296.2
	UTI89	-109273.4	30932.0	0.001	-184558.3	-33988.5
	UTI89 with Cipro	-36477.1	13141.0	0.719	-111762.0	38807.8
	UTI89 with TMP-SMX	-37944.0	95406.0	0.684	-113228.9	37340.9

2.4 Discussion

The results of our study provide evidence that both a urinary pathogen and antibiotics may have a causal role in the development of calcium urolithiasis. Exposure to a non-urease producing strain of *E. coli* was demonstrated to increase both CaOx crystal adherence and stone burden in DM Malpighian tubules. However, the overall stone burden was only significantly increased at the day 7 time point but failed to show a persistent elevation at the later 14-day time point. This may suggest that the impact of *E. coli* on stone formation is transient and can be further propagated or ameliorated depending on other conditions. Further investigation will be required in order to delineate the potential influence of other factors such has the duration, severity, and virulence of the *E. coli* infection, and other factors such as antibiotic exposure and composition and resilience of the microbiota.

Antibiotic exposure of DM flies with both ciprofloxacin and TMP-SMX was observed to have an even greater effect on stone formation than inoculation with *E. coli*. In addition, the effect of both antibiotics on stone burden was further increased at the later 14-day time point compared to the 7-day time point, demonstrating that antibiotic exposure had a durable long-lasting effect. TMP-SMX demonstrated a trend towards increased stone formation compared with ciprofloxacin, which is consistent with previous human epidemiological studies which demonstrated a higher relative risk of stone formation with TMP-SMX compared with ciprofloxacin[20]. Interestingly, treatment of MDCK cells with ciprofloxacin significantly increased CaOx crystal adherence; however, this same effect

was not demonstrated with TMP-SMX exposure. This suggests that both TMP-SMX and ciprofloxacin may be increasing stone formation through different mechanisms.

The observed effect of both ciprofloxacin and TMP-SMX on stone formation are striking given that DM flies were only exposed to a sub-inhibitory concentration of antibiotics over a short 3-day course on days 5-7 during both the 7 and 14-day assays. Sub-inhibitory concentrations of antibiotics were utilized in this study as it was thought this may further increase the effect of *E. coli* infection on stone formation. This was based on previous evidence which demonstrated that sub-inhibitory concentrations of antibiotics can potentiate bacterial virulence[28]. Further investigation with alternate concentrations and durations of antibiotic exposure may help to define the effect of antibiotics on stone formation.

Exposure of both DM flies and MDCK cells to a combination of both *E. coli* UTI89 and either ciprofloxacin or TMP-SMX failed to demonstrate a difference in both CaOx crystal adhesion and stone formation. This suggests that antibiotic exposure may only potentiate stone formation in the absence a urinary pathogen. Based on our data, it appears as though antibiotic exposure has a more profound effect on stone formation compared to urinary pathogen exposure. Stone formation following antibiotic exposure was observed to be significantly higher and result in a more durable effect than treatment with *E. coli.* This is consistent with epidemiological data which has demonstrated correlations between higher antibiotic use and the increasing prevalence of urolithiasis. This also suggests stone formation in humans may occur from antibiotics that are prescribed for non-urinary infections or when the duration of antibiotic treatment surpasses the presence of the urinary pathogen. This may have particular implications for the use of

prophylactic antibiotics which are commonly prescribed to prevent recurrent UTIs and are often given over long durations at lower doses.

This study provides further evidence which supports a role of antibiotic exposure and urinary pathogens on the formation of calcium-based stone disease; however, the exact mechanisms involved have yet to be elucidated. Antibiotics and urinary pathogens may exert their lithogenic effect through a number of potential mechanisms including alteration of the gut microbiota, and stimulation of a host inflammatory response[29]. It is likely that both these mechanisms influence one another through an interdependent relationship as the gut microbiota has been shown to affect the inflammatory immune response and oxidative stress in other disease states[30]. It has previously been demonstrated that the microbiota is significantly different in both *Drosophila* and humans with calcium-based urinary stones[7,31]. Alteration of the microbiota may have a negative effect on oxalate homeostasis by reducing *O. formigenes* colonization and increasing gut permeability[32]. This leads to increased oxalate absorption and subsequent renal oxalate excretion which results in hyperoxaluria and an increased propensity for stone formation[33-34]. Further research investigating the effect of urinary stone disease, urinary pathogens, and antibiotic exposure on the microbiota are required.

It has also been postulated that alteration of the host inflammatory response by both bacteria and antibiotics may potentiate stone formation[13]. Bacterial pathogens are well known to trigger an immune response resulting in the production of pro-inflammatory cytokines and ROS[35]. In addition, similar inflammatory responses, with the release of pro-inflammatory cytokines [tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)] and differentiation of monocytes into inflammatory M1 macrophages, have also

been observed following exposure to antibiotics such as ciprofloxacin and CaOx crystals[36,37]. The inflammatory response triggered by exposure to either a urinary pathogen or antibiotic exposure may result in renal tubule cell injury and consequently increased attachment of CaOx crystals. It has previously been demonstrated that antibiotic treatment induces renal tubular damage and that damaged renal tubule cells exhibit increased adhesion of CaOx crystals and susceptibility to urinary pathogens[38,39]. The complex interplay between urinary bacteria, antibiotics, and CaOx crystals resulting in alteration of the host immune response may represent a potential mechanism in the formation of calcium-based urinary stone disease and requires future investigation.

In addition, antibiotics may further potentiate stone formation through a number of other mechanisms. Crystals of antibiotic substrates within the urine may serve as a nidus of stone formation through heterogenous nucleation[40,41]. In addition, antibiotic induced tubular damage has also been shown to cause hypercalciuria which may further contribute to stone formation[40].

The major limitation of this study is that it is conducted in a non-vertebrate model; however, DM provide a practical, inexpensive and useful organism for investigating the pathophysiology of urinary stone disease. The DM Malpighian tubules are the functional equivalent of human convoluted tubules and have been well studied in regard to solute transport and excretion mechanisms[42]. In addition, the *Drosophila* genome has been extensively characterized and demonstrated to have significant similarities with the human genome; thus, providing a powerful tool for the use of genetic engineering techniques[1]. The DM model has previously been demonstrated to serve as a useful high-throughput platform in order evaluate the mechanistic pathways of stone formation and

potential therapeutic agents for lithiasis prevention[1,43-44]. The findings of our study utilizing the DM model can now subsequently be confirmed and further investigated in a murine model followed by human studies.

In conclusion, urinary stone disease is a common condition with a dramatically rising incidence and a high recurrence rate despite modern treatment and prevention strategies[3]. As a result, urinary stone disease represents a tremendous burden on the health care system[11]. Further understanding of the pathogenesis of stone disease is necessary. Specifically, investigating the effect of urinary pathogens, antibiotic exposure and alterations in the microbiota on urolithiasis may lead to the development of new prevention and treatment strategies. For instance, cultivation of a robust microbiota with adequate colonization of *O. formigenes* may prove to prevent stone formation, and as such potential strategies for microbiota focused therapy could include antibiotic stewardship, probiotic therapy, and fecal microbiome transplant (FMT)[45,46].

2.5 References

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Chapter 3

3 Defining the Relationship between Urinary Infection and Urolithiasis: A Novel Role for Osteopontin and Zinc Transport

There is mounting evidence that the formation of calcium-based stone disease may be impacted by urinary pathogens. Prior epidemiological studies have demonstrated a strong association between a history of both culture-proven UTIs and the development of stone disease. In addition, we have previously demonstrated that exposure to a non-urease producing strain of *E. coli* increases CaOx stone burden in a *Drosophila* model and enhances adherence of CaOx crystals to renal epithelial cells. However, the nature of the relationship between urinary bacteria and stone disease, as well as the potential mechanisms involved, have yet to elucidated. Osteopontin which is a known potent inhibitor of CaOx urolithiasis and Zn which is involved as an early nidus for the mineralization process of urinary stones; have both also been shown to be involved in bacterial pathogenesis. We aimed to further investigate the potential effect of a non-urease producing bacteria, and treatment with OPN and Zn on the adherence of CaOx crystals to renal epithelial cells.

3.1 Introduction

3.1.1 Role of Osteopontin

Osteopontin is a negatively charged glycoprotein that regulates both physiologic and pathologic mineralization within the body and has been shown to be a potent inhibitor of CaOx urolithiasis and play a complex role in urinary stone disease[1]. It is normal constituent of the urine, which is synthesized by the kidney and secreted into the urine by renal epithelial cells of the loop of Henle, the distal tubules and the renal papillae[2]. It has been demonstrated to inhibit all steps of stone formation including nucleation, aggregation and growth of CaOx crystals[3]. Furthermore, it inhibits the adherence of CaOx crystals to renal epithelial cells and directs CaOx crystallization towards COD, which is significantly less adherent to the epithelium[4,5]. Osteopontin knockout mice exhibit significantly increased CaOx stone formation in the setting of hyperoxaluria, but do not spontaneously develop stones[6]. In addition, clinical studies to date have been inconclusive regarding the role of OPN in urinary stone disease. Current data support an important and complex role of OPN in urolithiasis, however, the exact nature of this relationship has yet to be established.

Osteopontin is also intimately involved in both acute and chronic inflammatory processes. Pro-inflammatory cytokines, like TNF-α and IL-1β; as well as the activation of macrophages by lipopolysaccharide (LPS) have been shown to induce OPN gene expression and protein secretion[7]. Furthermore, OPN has been implicated in the

regulation of intestinal inflammation and it is thought to play a role in the maintenance of the intestinal microbiota by supporting intraepithelial lymphocyte survival[8-11]. Osteopontin knockout mice have been shown to have an altered gut microflora and an accelerated development of colitis[10,11]. Osteopontin has also been shown to have a protective effect in polymicrobial endodontic infections by reducing biofilm formation and increasing phagocyte recruitment[12,13]. The glycoprotein plays a large role in both urinary stone disease and the regulation of infectious and inflammatory processes, and there is growing evidence to suggest that urinary bacteria contribute to the pathogenesis of calcium urolithiasis. We propose a novel role for OPN in the development of stone disease, where urinary bacteria potentiate stone formation by altering the host inflammatory response through the regulation of OPN.

3.1.2 Role of Zinc Transport Proteins

Zinc is an essential trace element that has been implicated as an early nidus for the mineralization process of urinary stones. It was identified within Randall's plaques and urinary calculi in small amounts and is thought to contribute to the process of heterogeneous nucleation[14, 15]. Several studies utilizing *Drosophila* stone models have demonstrated a potential important role for Zn in urinary stone formation. A diet-induced CaOx *Drosophila* stone model revealed an up-regulation of Zn binding proteins with stone formation[14]. Furthermore, inhibition of Zn transporter proteins led to a suppression of stone formation[16].

Zinc is also known to play an integral role in both immune system function and bacterial pathogenesis[17, 18]. Exposure of dendritic cells to LPS alters the expression of several Zn transporters and decreases the intra-cellular availability of Zn[19]. It has also been shown that increased Zn levels decrease dendritic cell activation and inhibit the secretion of pro-inflammatory cytokines normally stimulated by LPS[19].

Zinc transport systems play an important role in pathogenesis of UTIs with *E. coli* through the modulation of virulence factor expression[14,20]. In addition, *E. coli* has been demonstrated to sequester Zn which may actually form the nidus for further stone formation[21]. Zinc is an essential element that plays a vital role in both host immunity and bacterial pathogenesis and has been implicated as an inciting event of stone formation. We hypothesize that urinary bacteria enhance CaOx stone formation by altering host Zn transport mechanisms.

3.2 Methods

3.2.1 Calcium Oxalate Crystal Adherence

Adherence of CaOx crystals to renal epithelial cells was determined with a crystal adherence assay utilizing MDCK renal epithelial cells (ATCC-CCL-34, NBL-2, Manassas, VA). MDCK cells were grown to 90% confluence on cell culture plates (172931, Thermo Fisher Scientific, Waltham, MA) in 5% CO_2 at 37°C in Dulbecco's Modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham,

MA). MDCK cells were exposed to UTI89 (10^3 CFU) for 20 minutes, and then incubated with CaOx crystal suspension (0.5 mg/mL) in artificial urine with or without the addition of OPN (0.1 μg/mL; SRP3131, Sigma Inc., Oakville, Canada), or Zn chloride (500 μg/mL; 229997; Sigma Inc., Oakville, Canada) for an additional 20 minutes. Unattached crystals were washed free with culture media and the adherence of CaOx crystals was determined with birefringence microscopy. The concentrations of OPN and Zn utilized were consistent with concentrations normally found in human urine[22,23]. Further details regarding the experimental protocol are elaborated on in section 2.2.2.

3.2.2 Data Analysis

Birefringent microscopy images of CaOx crystals adhered to the MDCK epithelial cells were obtained with an inverted light microscope (Nikon Inc., Tokyo, Japan) under a 100x magnification. MATLAB (MathWorks, 2018) was used to measure pixel intensity of birefringent CaOx crystals with a threshold pixel intensity of 150. Background pixel intensity was subtracted for all image analysis. A one-way analysis of variance (ANOVA) was utilized for statistical analysis with SPSS version 25.0 (IBM, New York, USA).

3.3 Results

3.3.1 Calcium Oxalate Crystal Adherence to Renal Epithelial Cells Following Treatment with *Escherichia coli* and Osteopontin

As previously demonstrated MDCK cells treated with UTI89 were shown to have a significantly increased adherence of CaOx crystals ($p<0.001$). However, exposure to OPN dramatically reduced adherence of CaOx crystals both in the control and UTI89 treated groups ($p<0.001$). The effect of OPN on CaOx crystal adherence in the control treatment is in keeping with OPNs known activity as a stone inhibitor. The ability of OPN to dramatically decrease CaOx crystal adhesion was consistent in the UTI89 treated group, demonstrating that OPN is able to negate the effect of *E. coli* exposure on CaOx crystal adherence. Representative birefringent microscopy images of CaOx crystal adherence to MDCK cells following treatment with *E. coli* UTI89 and OPN are shown in figure 18. In addition, the mean measured pixel intensity of the CaOx crystals stratified by treatment group is displayed in figure 19. Details of the ANOVA statistical analysis for all group comparisons are summarized in tables 15-17.

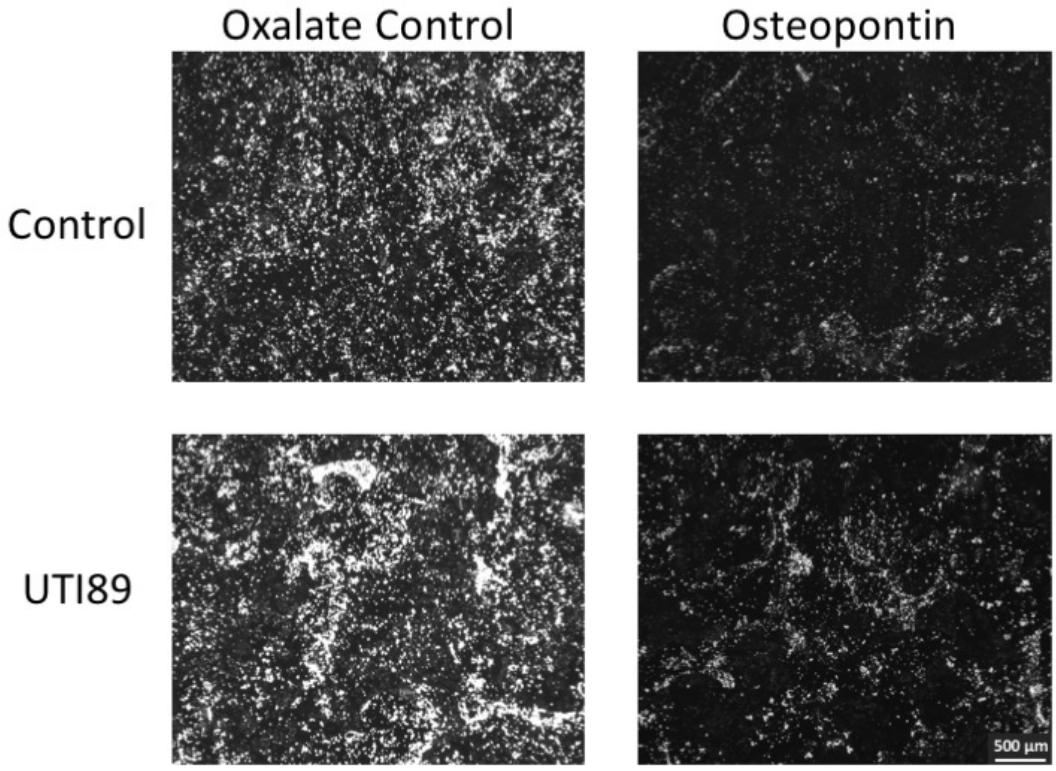

Figure 18: Representative birefringence microscopy images of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with *Escherichia coli* UTI89 (10^3 CFU) or osteopontin (0.1 µg/mL) under 100x magnification.

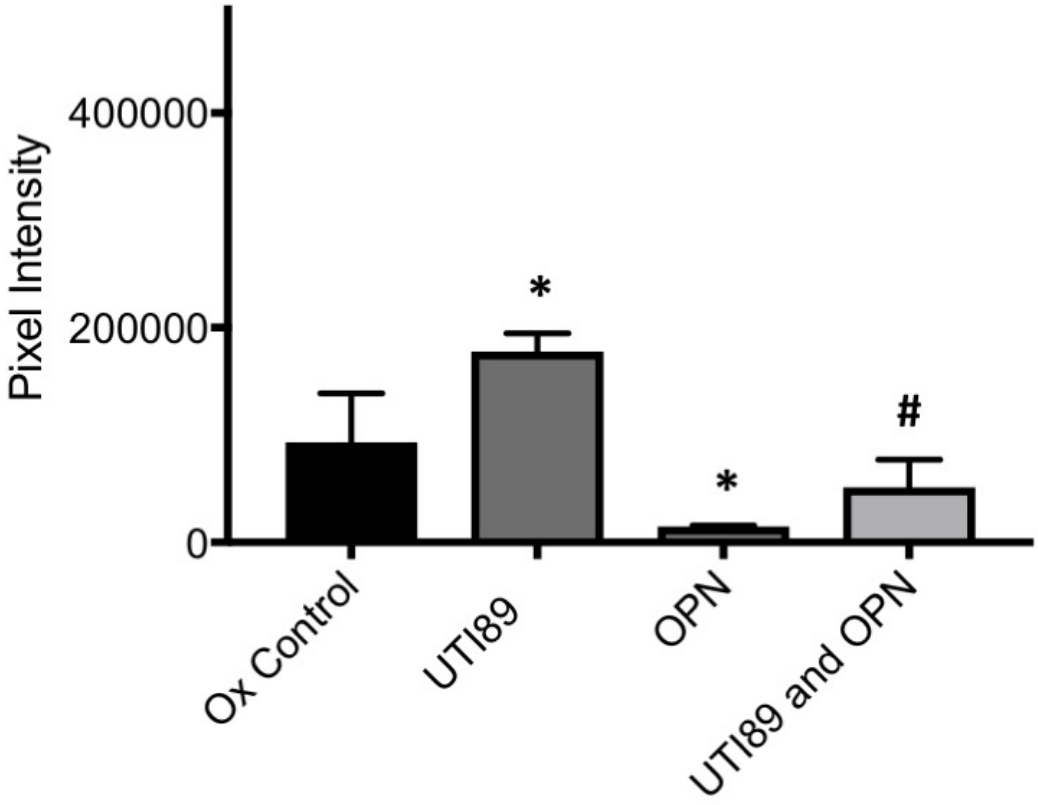

Figure 19: Measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with *Escherichia coli* UTI89 (10^3 CFU) or osteopontin (0.1 μg/mL). Data displayed as mean ± SEM (n=15/group), * $p<0.05$ compared with oxalate control., #$p<0.05$ compared with UTI89.

Table 15: Mean measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with *Escherichia coli* UTI89 or osteopontin (0.1 μg/mL).

					95% CI	
	N	Mean	SD	Std. Error	Lower	Upper
Oxalate Control	15	93528.9	45336.1	12116.6	67352.7	119705.2
UTI89	15	177679.7	17104.7	4416.4	168207.4	187151.9
OPN	15	14868.6	994.2	256.7	14318.0	15419.1
UTI89 and OPN	15	51556.5	25519.1	658.0	37424.4	65688.5

Table 16: ANOVA analysis of mean measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with *Escherichia coli* UTI89 or osteopontin (0.1 μg/mL).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.903E+11	5	9.806E+10	73.936	0.000
Within Groups	1.101E+11	84	1.326E+9		
Total	1.109E+12	89			

Table 17: ANOVA analysis with Tukey post-hoc analysis for multiple group comparison of mean measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with *Escherichia coli* UTI89 or osteopontin (0.1 μg/mL).

Group		Mean Difference	Std.	Sig.	95% CI Lower	95% CI Upper
Oxalate Control						
	UTI89	-84150.72	13533.6	0.000	-	-44668.3
	OPN	78660.4	15333.2	0.000	39177.9	118142.8
UTI89						
	UTI89 with OPN	126123.2	13298.2	0.000	87327.5	164918.9

3.3.2 Calcium Oxalate Crystal Adherence to Renal Epithelial Cells Following Treatment with *Escherichia coli* and Zinc Chloride

In addition to OPN, the potential role of Zn on the relationship between urinary pathogens and urolithiasis was also investigated. Treatment of MDCK cells with UTI89 resulted in significantly increased adherence of CaOx crystals ($p<0.001$). Exposure to Zn chloride significantly increased adherence of CaOx crystals both in the control and UTI89 treated groups ($p<0.05$). Co-treatment with both UTI89 and Zn appeared to have an additive effect of crystal adherence suggesting that Zn is acting to further enhance or propagate the effect of *E. coli* on CaOx crystal adherence. Representative microscopy images of CaOx crystal adherence to MDCK cells following treatment with *E. coli* UTI89 and Zn chloride are depicted in figure 20. In addition, the mean measured pixel intensity of the CaOx crystals stratified by treatment group is graphed in figure 21. Details of the ANOVA statistical analysis for all group comparisons are summarized in tables 18-20.

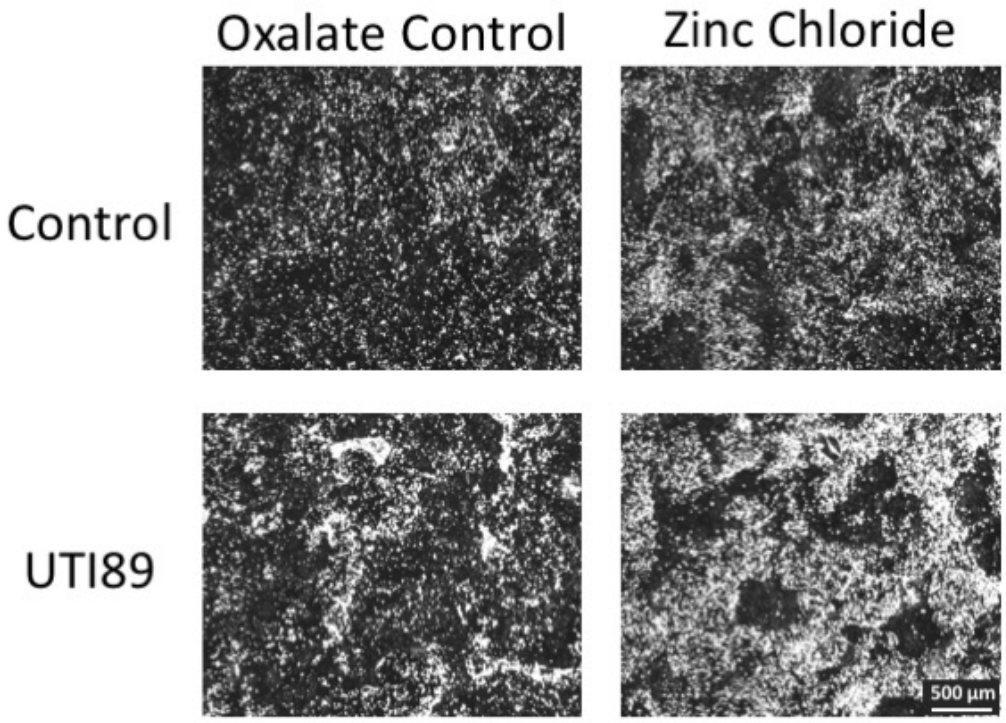

Figure 20: Representative birefringence microscopy images of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with *Escherichia coli* UTI89 (10^3 CFU) or zinc chloride (500 µg/mL) under 100x magnification.

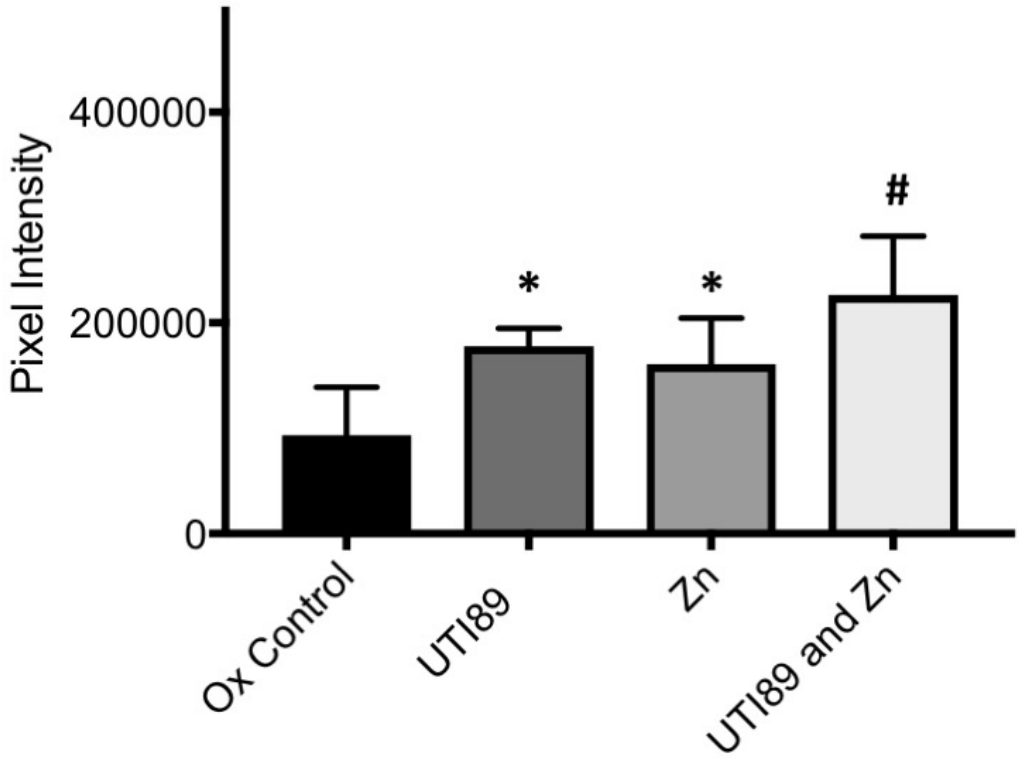

Figure 21: Measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells with *Escherichia coli* UTI89 (10^3 CFU) or zinc chloride (500 μg/mL). Data displayed as mean ± SEM (n=15/group), * p<0.05 compared with oxalate control., #p<0.05 compared with UTI89.

Table 18: Mean measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with *Escherichia coli* UTI89 or zinc chloride (500 μg/mL).

					95% CI	
	N	Mean	SD	Std. Error	Lower	Upper
Oxalate Control	15	93528.9	45336.1	12116.6	67352.7	119705.2
UTI89	15	177679.7	17104.7	4416.4	168207.4	187151.9
Zn	15	160855.9	43440.4	11216.3	136799.4	184912.3
UTI89 and Zn	15	226337.6	55881.9	14428.7	195391.2	257284.0

Table 19: ANOVA analysis of mean measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with *Escherichia coli* UTI89 or zinc chloride (500 μg/mL).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.903E+11	5	9.806E+10	73.936	0.000
Within Groups	1.101E+11	84	1.326E+9		
Total	1.109E+12	89			

Table 20: ANOVA analysis with Tukey post-hoc analysis for multiple group comparison of mean measured pixel intensity of calcium oxalate crystal adherence to MDCK renal epithelial cells following treatment with *Escherichia coli* UTI89 or zinc chloride (500 μg/mL).

Group Comparison		Mean Difference	Std. Error	Sig.	95% CI Lower	95% CI Upper
Oxalate Control						
	UTI89	-84150.72	13533.6	0.000	-123633.16	-44668.3
	Zn	-67326.9	16123.5	0.000	-106809.3	27844.5
UTI89						
	UTI89 with Zn	-48657.9	13298.2	0.006	-87453.7	-9862.2

3.4 Discussion

The results of this study provide evidence that urinary pathogens impact CaOx crystal adherence and that both Zn and OPN may be involved in this process with opposing effects. Exposure of a non-urease producing strain of *E. coli* to renal epithelia cells demonstrated a significant increase in CaOx crystal adherence. Both Zn and OPN significantly affected CaOx crystal adherence individually and in combination with UTI89. However, they were noted to have opposing effects with Zn increasing CaOx adherence compared with OPN which reduced crystal adherence.

Consistent with its known role as a stone inhibitor, OPN significantly decreased CaOx crystal adherence both on its own and in combination with the *E. coli.* It was observed to be very potent and dramatically reduce crystal adherence to renal epithelial cells. It almost completely reversed the accentuated crystal adherence observed with UTI89 exposure.

Osteopontin has previously been demonstrated to inhibit all steps of stone formation including nucleation, aggregation and growth of CaOx crystals[24]. In addition to this, it has also been observed to play an important role in inflammatory processes. The gene expression and protein secretion of OPN can be induced by multiple pro-inflammatory cytokines[25]. It is possible that one of the mechanisms that urinary pathogens use to potentiate stone disease is through modulation of the host immune response and subsequent inhibition of OPN. Osteopontin has been shown to play a complex role in

immune system function and further research is required to elucidate the ways in which OPN may interact with the inflammatory response to affect stone formation.

Contrary to the effects of OPN, Zn was observed to increase CaOx crystal adherence both individually and in combination with UTI89. It appeared to have an additive effect with the *E. coli,* further potentiating the observed increase in CaOx crystal adherence.

Zinc has been implicated as both a nidus for the mineralization of urinary stones and is known to be involved in both immune system function and bacterial pathogenesis. It is thought to serve as a nidus for stone formation through the process of heterogeneous nucleation[26]. In addition to this, several studies utilizing DM stone models have supported an important role for Zn in urolithiasis, with Zn binding proteins being up-regulated with stone disease, and genetic inhibition of Zn transport proteins resulting in a suppression of stone formation[26,27].

Along with this, increased Zn levels have been shown to down-regulate the secretion of pro-inflammatory cytokines and ROS which are normally stimulated by LPS[28,29]. Given the role of Zn in both host immunity and bacterial pathogenesis, as well stone formation, it may play an important role in the relationship between urolithiasis and urinary bacteria. This is further supported by our data which demonstrate that Zn increased CaOx crystal adherence and potentiated the effect of *E. coli*. This suggests that urinary pathogens may also increase stone formation through the alteration of Zn levels which may serve to affect the host immune response.

The major limitation of this study is that it is conducted in an *in vitro* model which focuses specifically on a single process in the complex cascade of events which occurs

during the formation of urinary calculi. However, this provides a practical, inexpensive and useful assay for investigating the pathophysiological mechanisms in urinary stone disease. It has previously been well described and utilized to study the mechanisms of urolithiaisis[31]. In addition, the adherence of CaOx crystals to renal epithelial cells represents a critical step in the formation of urinary calculi as without this the crystals would pass through the urinary system without difficulty, and adherence allows for further aggregation and growth of crystals[31]. Given that the potential mechanisms involved in the relationship between urinary pathogens and urolithiasis remain largely undefined this assay serves as a useful high-throughput platform in order to identify other potential compounds which may be involved for further investigation.

The influence of urinary pathogens on the formation of urinary calculi likely represents a complex relationship with multiple contributory mechanisms. Further investigation will be required in order to better delineate the exact role of both OPN and Zn and to characterize other potential contributing mechanisms.

3.5 References

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Chapter 4

4 General Discussion

This research showed that a urinary pathogen and antibiotics such as, ciprofloxacin and TMP-SMX, can have a direct impact on the formation of calcium-based stone formation. In addition, both OPN and Zn transport proteins may be involved in this process. However, the exact mechanisms involved remain poorly understood and require further elucidation.

4.1 Understanding the Relationship between Urinary Pathogens, Antibiotics and Stone Disease: Implications for the Host-Inflammatory Response and the Microbiota

Calcium urolithiasis is a complex pathophysiological process which is likely multifactorial in etiology and involves the interplay of multiple different mechanisms. A schematic diagram summarizing potential mechanisms involved in the relationship between urolithiasis, urinary pathogens and antibiotics exposure is depicted in figure 22. Future research should focus on better characterization of the host inflammatory response and the microbiota role in response to both antibiotics and pathogens.

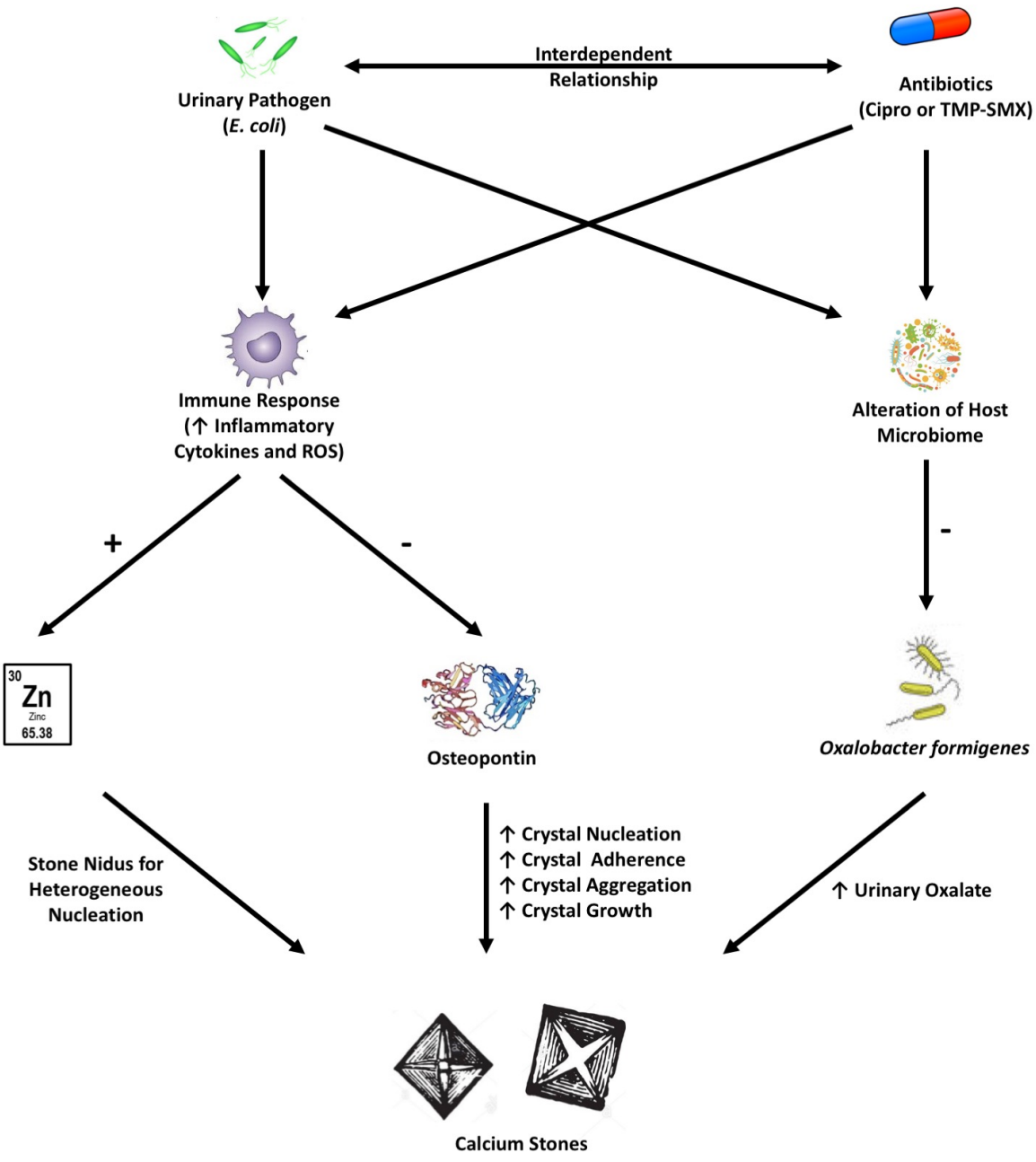

Figure 22: Schematic diagram of potential mechanisms involved in the relationship between a urinary pathogen, antibiotic exposure, osteopontin, zinc, and the formation of calcium-based stones.

4.1.1 The Microbiota and Oxalate Metabolism

The precise relationship between urinary bacteria, antibiotics and stone disease has yet to be determined; however, it has been postulated that both bacteria and antibiotics alter the host microbiota thereby potentiating stone formation. This is reflected in the gut microbiota being significantly different in stone forming patients and those with hyperoxaluria[1,2]. In the *Drosophila* model used here, the microbiota was also noted to be significantly different when CaOx stones were present compared to controls. Analysis with 16S rRNA sequencing demonstrated significantly increased levels of lactobacilli within the microbiota of *Drosophila* with CaOx urolithiasis[2]. This may actually represent a compensatory protective mechanism that occurs as a result of stone disease as many species of lactobacilli are known to degrade oxalate[2]. Previous work has demonstrated that certain oxalate metabolizing bacterial species are enhanced in the setting of hyperoxaluria[3]. This suggests a greater potential role for the microbiota in the pathogenesis of stone disease; however, a better understanding of the mechanisms involved is required.

While many different theories have been proposed, it is likely that antibiotics and pathogens alter the microbiota and reduce the ability of the gut to maintain oxalate homeostasis. In support of this, it has been demonstrated that changes to the microbiota within the small intestine and colon affect increased gut permeability. This would likely increase the absorption of oxalate and inflammatory bacterial components thereby having a further potential effect on the formation of stones. Furthermore, colonization with

beneficial bacteria can serve to reduce stone risk; for instance, it has previously been shown that gut colonization with *Oxalobacter* species promotes enteric oxalate secretion and reduces urinary oxalate secretion[4].

Further research is required to better delineate the relationship between stone disease and the gut microbiota and how this is influenced by antibiotic exposure. Future studies should focus on the influence of antibiotics on gut permeability and how this may be improved through the co-administration of probiotics. In addition, FMT with *O. formigenes* and other oxalate degrading bacterial species should be investigated as a potential therapeutic strategy to reduce urinary oxalate levels and thus the risk of stone disease. Given that enrichment of oxalate metabolizing species may occur as a compensatory mechanism for hyperoxaluria, increasing oxalate exposure to the microbiota within the lower gastrointestinal tract may also serve to increase oxalate degradation within the gut.

4.1.2 The Host Inflammatory Response

It has been hypothesized that the inflammatory response within the kidney is also altered by urinary bacteria and antibiotics providing a potential mechanism for enhancing stone formation[4]. Bacteria such as *E. coli* and *Proteus mirabilis* can reach the kidney when they cause pyelonephritis; however, studies suggest that they may also exist asymptomatically[5]. Bacterial pathogens are well known to trigger an immune response consisting of M1 macrophage activation which results in the production of pro-inflammatory cytokines such as IL-1β, IL-12, TNF-α, and ROS[6]. However, recent data have demonstrated that exposure of human monocyte cells to CaOx crystals results in a very similar response with the production of IL-1β, TNF-α, IL-6, and activation of M1 macrophages[7]. In addition, exposure of the monocyte cells to CaOx crystals followed by LPS, the major outer surface membrane component of gram-negative bacteria, resulted in an enhanced inflammatory cytokine expression compared with CaOx crystals alone[7].

Furthermore, bactericidal antibiotics such as fluoroquinolones, and β-lactams have been shown to directly induce the formation of ROS[8]. The inflammatory cascade triggered by exposure to either a urinary pathogen or antibiotics may result in renal tubular cell injury thereby increasing CaOx crystal adhesion to epithelial cells and subsequently increasing stone formation[9,10]. This evidence and our data suggests that alteration of the host immune response from both urinary bacteria and antibiotic exposure may be impacting the formation of CaOx stones.

It may be beneficial for future studies to focus on further examining the immune response and associated with stone disease as well as the exposure to antibiotics, potentially through next-generation RNA sequencing techniques. In addition, the production of ROS should also be examined under these conditions. The role of Zn on immune modulation in stone disease should also be further evaluated.

4.2 Potential Future Strategies

Evidence suggests that alterations in the host immune response may be an underlying mechanism in the pathophysiology of urolithiasis. Thus, a potential therapeutic strategy would include substances with anti-oxidant properties which serve to reduce oxidative stress mediated by ROS, formed as a result of exposure to urinary pathogens or antibiotics. Future study could examine inflammatory markers in humans following antibiotic exposure and whether or not this could be affected by the co-administration of non-steroidal anti-inflammatories.

A prior study demonstrated that the traditional Chinese medicinal plant, *Glechoma longituba*, reduced oxidative stress induced by CaOx in renal epithelial cells[11]. Additionally, a number of alkyl catechols which are compounds naturally found in fermented vegetables, ciders, and sourdough breads have been demonstrated to robustly activate oxidant defense pathways and have been shown to lower urinary oxalate levels through bacterial metabolism[12,13]. Consequently, anti-oxidant substances found in fermented foods may represent a potential treatment strategy for urolithiasis. Further

study assessing differences in the gut microbiota with fermented food intake may be enlightening with regards to their potential ability to reduce stone formation.

In addition, given the mounting evidence that the microbiota is implicated in the development of urolithiasis future strategies for the treatment and prevention of stone disease will potentially focus on the cultivation and maintenance of a healthy and robust microbiota which is resilient to stress and has the ability to recover following perturbation. For instance, strategies for promoting a beneficial microbiota could include antibiotic stewardship and facilitating dietary diversity which has shown to increase the robustness of the microbiota[14-17].

Another potential strategy would include the use of probiotics in order to reinforce or support the microbiota. This strategy could potentially work through multiple mechanisms including improving GI tract barrier function, reducing paracellular oxalate absorption, and increasing the metabolism and degradation of oxalate. Given the well-established role of *O. formigenes* in oxalate metabolism and its implication with human urolithiasis, human and *in vivo* studies have investigated the potential application of probiotic *O. formigenes* for the treatment of stone disease. These studies have demonstrated a significant reduction in urinary oxalate levels following administration of *O. formigenes* probiotic in both animal and human studies[18-21]. While this appears to be a potentially promising treatment, further study is required to determine the safety and efficacy[21-23].

Other therapeutic options directed at restoring the microbiota include both FMT and microbiome reconditioning which involves collecting and reconditioning a patient's own

microbiota to improve its overall oxalate degrading abilities and then reintroducing it to them. While the primary use of FMT is currently for *Clostridium difficile* infection, assessment of FMT against other diseases are currently underway[24-26]. It is thought that FMT may allow for a promising nephrolithiasis treatment as it could potentially restore intestinal barrier function and oxalate degradation and secretion within the gut. This would reduce urinary oxalate secretion thereby decreasing the risk of stone formation. FMT may lead to more durable results than microbiota modification with probiotics, as previous studies have demonstrated the propensity of gut microbiotas to revert back to their initial compositions following discontinuation of probiotic therapy[27]. Similar to FMT, microbiome reconditioning may allow for restoration of the microbiota without the potential risks of transmission of unwanted phenotypes or infectious agents that could occur with allogeneic transplant[24,26,27].

With the growing evidence supporting an important role of the microbiota in urinary stone disease there exist multiple potential promising treatment strategies. However, before these can be adopted, further investigation is required to better define the exact mechanistic role of the microbiota in stone disease and how this can be altered to reduce stone formation.

4.3 Final Conclusion

Urinary stone disease is a common urological condition with a dramatically rising incidence among all age groups[28]. Despite modern treatment and prevention strategies urolithiasis continues to have a high recurrence rate thus resulting in considerable patient

morbidity and a tremendous burden on the health care system[29]. An improved understanding of the pathophysiology underlining urolithiasis is paramount and should focus on the potential alteration of the host inflammatory response and microbiota in urinary stone disease. The present thesis suggests that further investigation into how disruption of urinary and gut microbes occurs, including through antibiotic administration, could be revealing. This perturbation may then lead to damaged gut barrier function, inflammation, and propagation of non-oxalate degrading organisms which may result in increased stone formation. Furthermore, this research may help to identify potential new therapeutic targets and strategies for urolithiasis, including antibiotic stewardship, anti-oxidants, probiotics, and microbiome modification.

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Appendices

Appendix A1: Abbreviations

ANOVA	Analysis of variance
CaOx	Calcium Oxalate
CFU	Colony forming unit
CO_2	Carbon dioxide
COD	Calcium oxalate dihydrate
COM	Calcium oxalate monohydrate
DM	*Drosophila melanogaster*
DMEM	Dulbecco's Modified Eagle Media
E. coli	*Escherichia coli*
FBS	Fetal bovine serum
FMT	Fecal microbiome transplant
H. pylori	*Helicobacter pylori*
IL-1β	Interleukin-1 beta
LPS	Lipopolysaccharide
MDCK	Madin-Darby Canine Kidney
NaOx	Sodium Oxalate
O. formigenes	*Oxalobacter formigenes*
OPN	Osteopontin
PBS	Phosphate buffered saline
PTH	Parathyroid hormone
ROS	Reactive oxygen species
TMP-SMX	Trimethoprim-sulfamethoxazole
TNF-α	Tumor necrosis factor-alpha
UTI	Urinary tract infection
Zn	Zinc

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